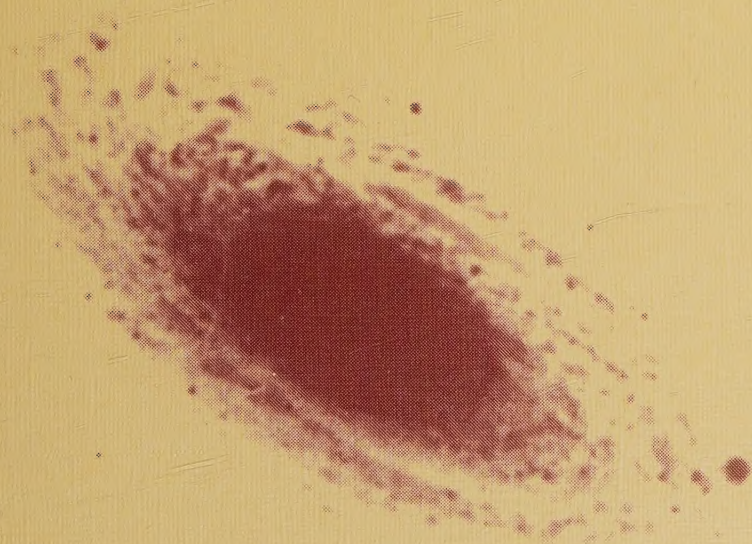


NMR STUDIES OF ION BINDING TO PROTEINS

THOMAS ANDERSSON



Lund 1981

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Abstract The <u>ion binding</u> properties of <u>proteins</u> were studied by NMR methods. Ion binding to <u>cytochrome c</u> was studied by ^{35}Cl NMR and ^{23}Na NMR. Both oxidation states were found to bind anions such as Cl^-, ClO_4^-, phosphate and iron hexacyanides. No indications of Na^+ binding could be detected. The ion binding properties are somewhat different in the two forms. Ca^{2+} binding to <u>phospholipase A₂</u> was studied using ^{43}Ca NMR. Binding constants, exchange rates and apparent pK values were determined. Significant differences between the zymogen and the active enzyme were found. Cation binding to <u>troponin C</u> was studied by ^{43}Ca NMR and ^{25}Mg NMR. The exchange rate of one of the two regulatory sites was affected by Mg^{2+} binding to the weak sites. Ca^{2+} binding to <u>calmodulin</u> was studied by ^{43}Ca NMR and ^{113}Cd NMR. The two weaker sites were shown to have exchange rates differing by a factor of ca. 30. <u>Trifluoperazine</u> binding to troponin C and calmodulin was studied by ^{43}Ca and ^{113}Cd NMR. Cation binding to <u>Panulirus interruptus hemocyanin</u> was studied by ^{23}Na and ^{43}Ca NMR.			
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NMR STUDIES OF ION BINDING TO PROTEINS

Thomas Andersson

Lund 1981

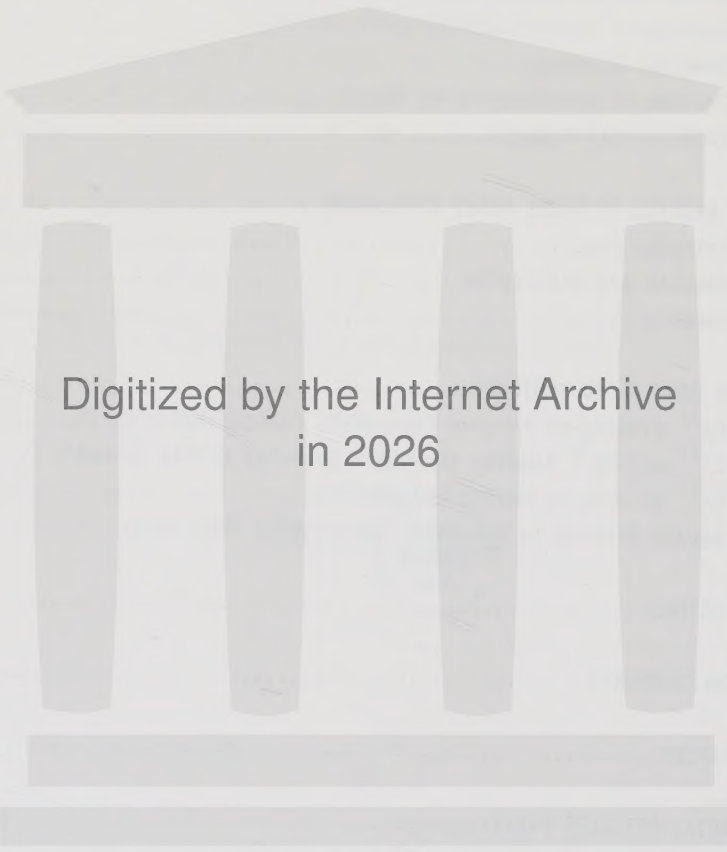
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1. PRESENTATION

This thesis is a review of the results and conclusions from previous work described in Articles I-VII listed below. Some unpublished results are included in order to present a more complete picture of the field.

- I. "Ion Binding to Cytochrome c Studied by Nuclear Magnetic Quadrupole Relaxation"; Thomas Andersson, Eva Thulin and Sture Forsén (1978) Biochemistry 12, 2487-2493.
- II. "Perchlorate Binding to Cytochrome c"; Thomas Andersson, Jonas Ångström, Karl-Erik Falk and Sture Forsén (1980) Eur. J. Biochem. 110, 363-369.
- III. "Calcium Binding to Porcine Pancreatic Prothospholipase A₂ Studied by ⁴³Ca NMR"; Thomas Andersson, Torbjörn Drakenberg, Sture Forsén Tadeusz Wieloch and Mats Lindström (1981) FEBS Lett. 123, 115-117.
- IV. "A ⁴³Ca and ²⁵Mg NMR Study of Rabbit Skeletal Muscle Troponin C"; Thomas Andersson, Torbjörn Drakenberg, Sture Forsén and Eva Thulin (1981) FEBS Lett. 125, 39-43.
- V. "Characterization of the Ca²⁺ Binding Sites of Calmodulin from Bovine Testis"; Thomas Andersson, Torbjörn Drakenberg, Sture Forsén and Eva Thulin, manuscript.
- VI. "Direct Observation of ⁴³Ca NMR Signals from Ca²⁺ ions Bound to Proteins"; Thomas Andersson, Torbjörn Drakenberg, Sture Forsén, Eva Thulin and Marianne Swärd, J. Am. Chem. Soc. in press.
- VII. "Characterization of Cation Binding to Panulirus interruptus Hemocyanin by ⁴³Ca and ²³Na NMR"; Thomas Andersson, Emilia Chiancone and Sture Forsén, submitted for publication.

2. INTRODUCTION

Metal ions have long been known to play specific essential roles in biological systems. They stabilise the structure of macromolecules, they catalyse chemical reactions, they affect the binding of small molecules and they participate in redox reactions. They can also induce conformational changes in proteins which in turn will modulate enzymatic activity or for instance, control muscular contraction.

Metals such as Fe, Cu and Co are often involved in redox reactions and oxygen storage; Zn^{2+} ions are often an integral part of the active sites of enzymes. The metal ions are bound to the protein via nitrogen and sulphur atoms and show little or no chemical exchange with the environment. This type of protein is often referred to as a metallo-protein or metallo-enzyme.

Another group of proteins is the metal-activated or metal-stabilised proteins. These proteins form weak to moderately strong complexes with alkaline earth or alkali metal ions largely through electrostatic interactions with oxygen atoms in amino acid side chains or in the polypeptide chain. These ions may serve either as catalytic units in enzymes or as structural elements in proteins.

The possible role of simple anions such as chloride, phosphate and bicarbonate is less well documented. There are however a few reported cases of activation of enzymes by anions.

The study of ion binding to proteins has been hampered by the fact that ions such as Na^+ , K^+ , Mg^{2+} , Ca^{2+} , HPO_4^{2-} and Cl^- are "silent" in most spectroscopic techniques but nuclear magnetic resonance has proved to be a useful tool in the study of protein-ion interactions.

NMR spectra often contain a great deal of detailed information about molecular structures, molecular interactions and molecular motion. NMR is however an inherently insensitive spectroscopic technique. In recent years, however, instrumental developments (particularly Fourier-transform (FT) methods and the use of cryomagnets with high magnetic

fields) have made the method useful in the study of biological problems.

There are two main types of approach to the study of ion-macromolecule interactions. The most common is to study a high resolution ^1H or ^{13}C NMR of the protein molecule and observe spectral perturbations produced on ion binding. A second and less common approach is to study the NMR signals of the ions interacting with the protein. The applicability of this latter method has been limited by the low NMR sensitivity of ions such as $^{25}\text{Mg}^{2+}$, $^{39}\text{K}^{+}$ and $^{43}\text{Ca}^{2+}$. In some cases ion binding to proteins can be studied indirectly by observing the NMR signal of another nucleus of better NMR suitability. This situation is illustrated in Figure 1 for the case of Ca^{2+} binding to proteins.

HIGH-RESOLUTION NMR STUDY OF THE PROTEIN

Observation of spectral perturbations produced on binding calcium (or lanthanides).

NMR STUDY OF THE INTERACTING IONS

- a) Direct study of the Ca^{2+} NMR signal.
- b) Indirect study of the calcium binding by 23-sodium NMR. Competition.
- c) Substitution of firmly bound calcium ions by a $I=1/2$ nucleus such as 113-cadmium.

Figure 1. Different approaches to the NMR study of Ca^{2+} binding to proteins.

In this project the binding of both anions and cations has been studied using all of the approaches described above. The main emphasis has however been on use of NMR studies of quadrupolar ions.

3. THEORETICAL AND EXPERIMENTAL SECTION

Table 1 shows the relevant nuclear properties of the most common simple ions in biological systems. All of these nuclei have spin quantum numbers $I > \frac{1}{2}$ and thus have a non-spherical charge distribution which can be described in terms of a nuclear electric quadrupole moment (eQ). If the charge distribution around the nucleus has less than cubic symmetry an electric field gradient (eq) is formed. This field gradient can couple to the electric quadrupole moment. Fluctuations in the magnitude and/or direction of the electric field gradients will therefore provide a relaxation mechanism for the nuclear magnetization. This relaxation mechanism (quadrupole relaxation) is so efficient that in most cases it is the only mechanism that needs to be considered [1].

Table 1. NMR properties of ions common in biological systems (from ref. 2).

	^{23}Na	^{25}Mg	^{35}Cl	^{37}Cl	^{39}K	^{43}Ca
Nuclear spin (I)	3/2	5/2	3/2	3/2	3/2	7/2
NMR frequency (ν) at 6 T (MHz)	67.6	15.6	25.0	20.8	11.9	17.2
Natural abundance (%)	100.0	10.1	75.5	24.5	93.2	0.15
Relative sensitivity (proton=1)	$9.3 \cdot 10^{-2}$	$2.7 \cdot 10^{-3}$	$4.7 \cdot 10^{-3}$	$2.7 \cdot 10^{-3}$	$5.1 \cdot 10^{-4}$	$6.4 \cdot 10^{-3}$
Quadrupole moment (10^{-24}cm^2)	0.14	0.22	-0.079	-0.062	0.11	-0.05

3.1. Quadrupole Relaxation

The magnetic relaxation of a spin $I > 1$ nucleus is generally not a single exponential decay [3]. For nuclei with half integer spin the decay of the transverse ($\alpha = 2$) and longitudinal ($\alpha = 1$) magnetization will be a weighted sum of $I + \frac{1}{2}$ exponentials [3].

$$M_1(t) = M_1(\infty) [1 - K (\sum_{i=1}^j C_{1,i} \exp(-R_{1,i} t))] \quad (1)$$

$$M_2(t) = M_2(0) [\sum_{i=1}^j C_{2,i} \exp(-R_{2,i} t)] \quad (2)$$

where $j = I + \frac{1}{2}$, $\sum_{i=1}^j C_{\alpha,i} = 1$ and $K = 2$ in the inversion recovery experiment [4].

The efficiency of the relaxation depends on the quadrupole coupling constant (χ), which is a product of the magnitude of the quadrupole moment (eq) of the nucleus in question and the magnitude of the fluctuating field gradients (eq). The relaxation rate is also dependent on the time scale of the fluctuations of the field gradients. This is described by the spectral density $F(\omega, \tau_c)$, which is a function of the correlation time τ_c , a time constant characterising changes in the magnitude or direction of the electric field gradients, and by the resonance frequency, ω , in radians/s.

$$R_{\alpha,i} = \text{const} \cdot \chi^2 \cdot F(\omega, \tau_c) \quad (3)$$

$$\chi = (e^2 q Q / h) \cdot (1 + \eta^2 / 3)^{\frac{1}{2}} \quad [\text{Hz}]$$

η is termed the asymmetry parameter and describes the deviations from cylindrical symmetry of the electric field gradients. It is generally assumed that $\eta=0$ and in practice this is a very good approximation.

A single exponential decay of the magnetisation is obtained when the relaxation rates of the different components are similar and/or when the amplitude of one of the components becomes dominant.

3.1.1. Quadrupole relaxation of spin $I=3/2$ nuclei

The magnetic relaxation of a $I=3/2$ nucleus is, as noted above, a weighted sum of two exponentials [2,3]

$$M_1(t) = M_1(\infty) [1 - K(0.2 \exp(-R_{1,1} t) + 0.8 \exp(-R_{1,2} t))] \quad (4)$$

$$M_2(t) = M_2(0) [0.6 \exp(-R_{2,1}t) + 0.4 \exp(-R_{2,2}t)] \quad (5)$$

$$\text{where} \quad R_{1,1} = \frac{2\pi^2}{5} \cdot \chi^2 \cdot \frac{\tau_c}{1 + (\omega\tau_c)^2} \quad (6)$$

$$R_{1,2} = \frac{2\pi^2}{5} \cdot \chi^2 \cdot \frac{\tau_c}{1 + (2\omega\tau_c)^2} \quad (7)$$

$$R_{2,1} = \frac{\pi^2}{5} \cdot \chi^2 \cdot \left[\tau_c + \frac{\tau_c}{1 + (\omega\tau_c)^2} \right] \quad (8)$$

$$R_{2,2} = \frac{\pi^2}{5} \cdot \chi^2 \cdot \left[\frac{\tau_c}{1 + (\omega\tau_c)^2} + \frac{\tau_c}{1 + (2\omega\tau_c)^2} \right] \quad (9)$$

The bi-exponential decay of the longitudinal magnetisation has proven difficult to detect due to the low intensity (20%) of one of the components but also in part due to the fact that the relaxation rates of the two components differ very little. For the transverse magnetisation the situation is much more favourable and the high resolution spectrum of a spin $I=3/2$ nucleus will, under non-extreme narrowing conditions, as a first approximation (see below) be a superposition of two Lorentzians having the same resonance frequency (see section 3.1.1), a broad signal accounting for 60% of the total intensity (line width at half height $\Delta\nu_{\frac{1}{2}} = R_{2,1}/\pi$) and a narrow signal for 40% of the intensity ($\Delta\nu_{\frac{1}{2}} = R_{2,2}/\pi$).

From Equations (4) - (9) it can be seen that for correlation times such that $\omega\tau_c \ll 1$ (extreme narrowing conditions) the relaxation rates of the two different components in the transverse and longitudinal relaxation have the same value. This results in single-exponential decays of the transverse and longitudinal magnetisation having the same time constants:

$$R_2 = R_1 = \frac{2\pi^2}{5} \cdot \chi^2 \cdot \tau_c \quad (10)$$

For correlation times such that $\omega\tau_c \approx 1$ the relaxation appears to be exponential. An important difference however is that R_1 and R_2 are no

longer equal. Bull et al. have derived approximate equations describing the apparent relaxation rates for $\omega\tau_c$ values less than 1.5 [5]:

$$R_1 = \frac{2\pi^2}{5} \cdot \chi^2 \cdot \left[\frac{0.2\tau_c}{1+(\omega\tau_c)^2} + \frac{0.8\tau_c}{1+(2\omega\tau_c)^2} \right] \quad (11)$$

$$R_2 = \frac{\pi^2}{5} \cdot \chi^2 \cdot \left[0.6\tau_c + \frac{\tau_c}{1+(\omega\tau_c)^2} + \frac{0.4\tau_c}{1+(2\omega\tau_c)^2} \right] \quad (12)$$

3.1.2. Quadrupole relaxation of spin I=7/2 and I=5/2 nuclei

The magnetic relaxation of a spin 7/2 nucleus according to Equations (1) and (2) is a sum of 4 exponentials. The situation is in principle analogous to the spin 3/2 case described above but a considerable complication is that the pre-exponential factors $C_{\alpha,i}$ (i.e. the intensities of the different components) are not constant. It is not possible to obtain analytical solutions of the relaxation equations but they can be solved numerically for each value of ω and τ_c [6]. When extreme narrowing conditions, $\omega\tau_c \ll 1$, apply the intensity of the slowest relaxing component is unity and:

$$R_1 = R_2 = \frac{3\pi^2}{10} \cdot \frac{(2I+3)}{I^2(2I-1)} \cdot \chi^2 \cdot \tau_c \quad (13)$$

When extreme narrowing conditions do not apply the relaxation becomes multiexponential. The decay of the longitudinal magnetisation can be approximated by a single exponential for correlation times such that $\omega\tau_c \leq 10$, since $C_{1,1} > 0.96$ for $\omega\tau_c \leq 10$ [6]. The decay of the transverse magnetisation is more complex and for $\omega\tau_c$ values greater than one, multiexponential behaviour is expected (see below).

Halle and Wennerström [7] have shown, by a perturbation treatment, that there are simple analytical expressions of the apparent relaxation rates that are valid up to $\omega\tau_c \leq 1$. The equations are identical to those derived by McLachlan [8] for the average relaxation rates $\langle R_1 \rangle$ and $\langle R_2 \rangle$ and for the case of quadrupolar relaxation are:

$$\langle R_1 \rangle = \frac{3\pi^2}{10} \cdot \chi^2 \cdot \frac{(2I+3)}{I^2(2I-1)} \left[\frac{0.2 \tau_c}{1+(\omega\tau_c)^2} + \frac{0.8 \tau_c}{1+(2\omega\tau_c)^2} \right] \quad (14)$$

$$\langle R_2 \rangle = \frac{3\pi^2}{10} \cdot \chi^2 \cdot \frac{(2I+3)}{I^2(2I-1)} \left[0.3 \tau_c + \frac{0.5 \tau_c}{1+(\omega\tau_c)^2} + \frac{0.2 \tau_c}{1+(2\omega\tau_c)^2} \right] \quad (15)$$

The situation for spin $I=5/2$ nuclei is completely analogous to the spin $7/2$ case.

3.1.3. Dynamic Frequency Shifts

In the practical application of the theory of quadrupole relaxation second order effects have usually been neglected. The theory predicts however a dynamic frequency shift which in general is different for the various components of the decay of the transverse magnetisation. As a result, the line shapes become asymmetric and shifted away from the resonance frequency ω_0 . For $\omega\tau_c \ll 1$ the dynamic frequency shifts are negligible [9,10].

$I = 3/2$: Even at $\omega\tau_c = 0.5$ the NMR signal is asymmetric. This asymmetry has often been overlooked since compensating phase adjustments will obscure the effect and thus produce errors (of the order of 1-5 %) in the relaxation rates obtained from a spectrum analysis.

$I = 5/2$ and $7/2$: Calculations on the NMR signal line-shape (H. Wennerström, private communication) shows that the NMR signals are asymmetric for $\omega\tau_c$ values greater than 0.5 ($\omega = 1 \cdot 10^8 \text{ s}^{-1}$, $\chi = 1-3 \text{ MHz}$). It can however be shown that it will be difficult to detect deviations from Lorentzian line-shape for $\omega\tau_c$ values less than 1.5.

3.2. Chemical Exchange

The low sensitivity of the quadrupolar ions (Table 1) together with a very efficient quadrupole relaxation makes the direct observation of the NMR signals from the "protein-bound" ions extremely difficult. The use of "ion-NMR" in the study of protein-ion interactions is however possible if there is a reasonably fast chemical exchange between "free" ions and bound ions.

3.2.1. Fast Chemical Exchange

If the rate of the chemical exchange, k_{OFF} , between the protein binding sites and the bulk solution is much faster than the relaxation rates at the protein binding sites, $k_{OFF} \gg R_2, R_1$, the observed relaxation rate is a weighted sum over the different sites the ions occupy:

$$R_{\alpha}^{OBS} = p_F R_{\alpha}^{FREE} + \sum_n p_{B,n} R_{\alpha,n} \quad (16)$$

where $p_{B,n}$ is the fraction of ions bound to the n 'th site, $R_{\alpha,n}$ and R_{α}^{FREE} are the relaxation rates for the ions bound to the n 'th site and for the free ions respectively. The population in the bulk solution is denoted p_F . The intrinsic relaxation rates are given by the expressions in the previous section.

In the case of "non-exponential" relaxation the situation readily becomes complex. Let us for convenience consider a two-site situation with one site holding the bulk solution ("extreme-narrowing") and the other being the protein binding sites (multi-exponential relaxation). The observed NMR signal then consists of a superposition of Lorentzians (with the same resonance frequencies to a first approximation) each having the line widths at half height:

$$\Delta\nu_i = (1-p_B)R_2^{FREE}/\pi + p_B R_{2,i}/\pi \quad (17)$$

Information obtainable:

The fraction of ions bound to a class of sites, p_B , can be expressed in terms of a binding model. For the case of site-binding, p_B is given by mass law considerations. For example if the total concentration of the ion is varied while the protein concentration remains constant, the association constant can be obtained from a fit of a chemical equilibrium model to the data. However the lowest available concentration in this type of study sets an upper limit for the binding constants determinable (typically 10^2 - 10^3 M^{-1}). Competition experiments and variable pH can provide additional information about the binding. It should be noted that these binding constants are apparent values since the concentration dependence of the activity coefficients is not known. Due to this difficulty the activity of the ions and proteins have been assumed to be equal to their respective concentrations.

Changes in the protein conformation may be detected as changes in p_B and/or as changes in the intrinsic relaxation rates, $R_{\alpha,n}$. Measurements of R_1 and R_2 provide information on the correlation time.

3.2.2. Intermediate Chemical Exchange

If the chemical exchange rate and the relaxation rate on the protein site are of the same order of magnitude both parameters affect the relaxation rate.

Due to the inherent low NMR-sensitivity of the quadrupolar ions the majority of ion binding studies have been made using a large excess of the ion in question. When there is chemical exchange the line width in both the bulk solution and in the protein sites are significantly changed. In this type of study however, the effect of the exchange rate on the "bulk" site is usually observed since this is much more easily measured experimentally. In a situation where the conditions above are met the observed relaxation rate is given by the "Swift-Connick" equation, given below for the special case of no chemical shift between the bulk site and the protein sites [11]:

$$R_{\alpha}^{OBS} = p_F R_{\alpha}^{FREE} + \sum_n \frac{p_{B,n}}{R_{\alpha,n}^{-1} + k_{OFF,n}^{-1}} \quad (18)$$

This simple equation, as mentioned above, is only valid for $p_F \gg p_B$, a situation which until now has been the normal case. However, with recent instrumental developments this condition is satisfied much less often.

For large values of p_B chemical exchange produces distortions of the Lorentzian line shape, except for very low and very high exchange rates. In such cases a total band-shape analysis is needed to obtain information about k_{OFF} , $R_{2,B}$ and p_B . The complete band-shape may be derived from the modified Bloch equations due to McConnell[12].

Information obtainable:

In the work presented in this thesis (Articles III-V), we have used simultaneously about 10 digitised NMR spectra at different temperatures and [metal ion]/[protein] ratios and iterated the parameters (p_B , $R_{2,B}$ and k_{OFF}) to find the best fit between calculated and experimental spectra (Drakenberg et al., to be published). To give a visual representation of the results of the fitting procedure the line widths at half intensity for the calculated spectra are shown as a solid line in the figures.

In practice it is necessary to measure both temperature dependence and ion-concentration dependence to get reliable estimates of the binding constant, the exchange rate and the quadrupole coupling constant.

The temperature dependence of the NMR signal line shape needs a little extra consideration. Figure 2 shows the temperature dependence of the line width at half intensity for a two-site case. The temperature dependence is in principle due to two main contributions (p_B is assumed to be constant), the exchange rate and the relaxation rate of the ion on the protein site. The latter is a function of the correlation time.

$$k_{OFF} = \frac{kT}{h} \exp(-\Delta G^{EX}/RT) \quad (19)$$

$$\frac{1}{\tau_c} = \frac{kT}{h} \exp (-\Delta G^{RE} / RT) \quad (20)$$

where k and h denote the Boltzmann and Planck constants respectively. In Figure 2 these two contributions are shown as broken and dotted lines respectively and the resulting line width are represented as a solid line.

In some of the Ca^{2+} binding studies the observed NMR line widths are affected by two classes of sites with exchange rates differing by one or two orders of magnitude. This will give a slightly changed temperature profile. Figure 3 shows an example of this situation where we have two equally populated protein sites with exchange rates differing by a factor of 50 at room temperature. The separate contributions from each class of site are shown as broken and dotted lines.

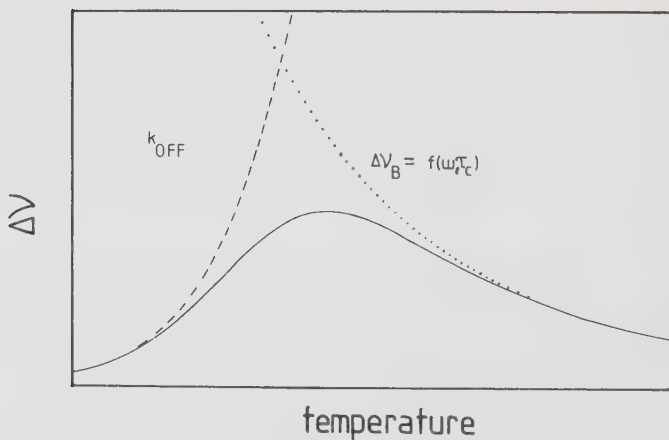


Figure 2. The temperature dependence of the line-broadening of the NMR signal of the "free" ions. The variations in the exchange rate and intrinsic line width are shown as broken and dotted lines respectively.

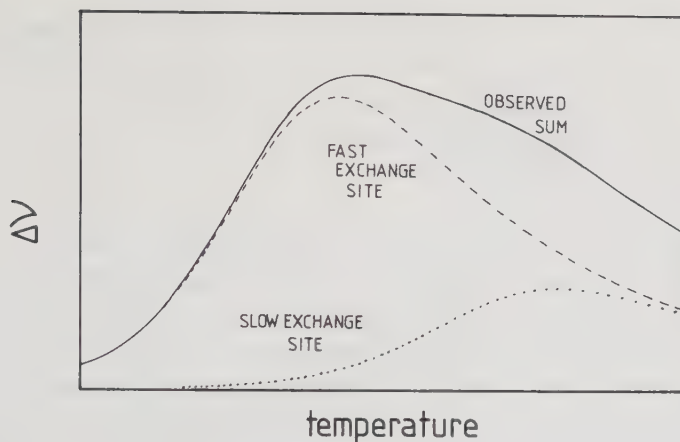


Figure 3. The temperature dependence of the observed line broadening (solid line) of the "free" ions for the case when the protein contains two ion binding sites, equally populated, with exchange rates differing by a factor of approximately 50 at room temperature.

3.2.3. Slow Chemical Exchange

When the exchange rate k_{OFF} is much slower than the intrinsic relaxation rate $R_{2,B}$ the observed NMR signal is simply a superposition of the signals from the "free" and "protein-bound" ions, each with an intensity corresponding to the respective population. The quadrupole relaxation method is usually inapplicable under these conditions because the NMR signal from the "protein-bound" ions generally escapes detection. This is a result of the low sensitivity of the quadrupole nuclei and the effective relaxation mechanism which produces line widths of the order of 10^4 - 10^5 Hz. This describes the general situation. ^{43}Ca is to be regarded as an exception.

^{43}Ca has been considered to be one of the "worst" ions from a sensitivity point of view, Table 1. The use of high magnetic fields and the use of isotopically enriched compounds changed the situation dramatically and made ^{43}Ca measurements possible at millimolar concentrations.

Due to the low quadrupole moment, ^{43}Ca gives "protein-bound" signals with line widths of $6\text{--}9 \cdot 10^2$ Hz for the Ca^{2+} binding proteins studied in Article VI. Measurements of the longitudinal relaxation rate and the signal line width can be used to obtain the quadrupole coupling constant and the correlation time.

This type of study is generally limited to conditions such that the concentration of "free" ions is negligible. Otherwise the NMR signal from the "free" ions will dominate the spectrum. This restricts us in practice to ^{43}Ca signals from Ca^{2+} binding sites with affinities greater than $10^5\text{--}10^6 \text{ M}^{-1}$. The method described above is extremely time consuming and expensive since considerable amounts of protein and enriched Ca^{2+} are needed. An alternative and complementary method for the study of Ca^{2+} binding sites of high affinity is to replace Ca^{2+} by a spin $I = 1/2$ nucleus.

3.3 The Use of Non-Quadrupolar Nuclei

As noted above replacement of the "native" metal ion by a spin $I = 1/2$ nucleus such as ^{113}Cd , ^{199}Hg or ^{207}Pb can provide information for cases where the use of quadrupolar ions is not applicable. The NMR signals are relatively narrow compared to those of the quadrupolar ions.

The use of the ^{113}Cd isotope has been shown to be of particular interest for the study of Zn^{2+} enzymes and Ca^{2+} binding proteins [2]. The chemical shift range of $^{113}\text{Cd}^{2+}$ is ca. 800 ppm. The shift of the ^{113}Cd NMR signal is very sensitive to the nature of the ligands (Figure 4) and in addition is sensitive to the coordination geometry. This makes ^{113}Cd a useful probe for protein conformational changes.

For Ca^{2+} binding proteins ^{113}Cd NMR can give information about the kinetics of the metal binding. For sites having metal ion exchange rates, k_{OFF} , which are slow compared to the chemical shift difference $\delta\omega$ (s^{-1}) between the protein sites and the bulk solution "slow exchange" conditions apply. In this case the observed ^{113}Cd spectrum consists of a superposition of the resonances from the different sites. In a 6-Tesla spectrometer this situation is generally met for exchange rates

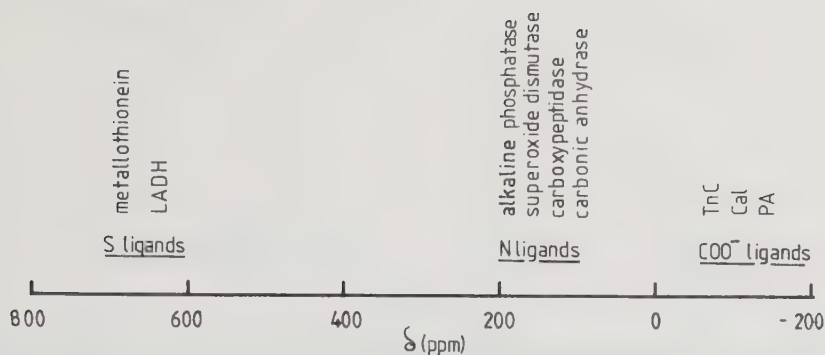


Figure 4. The chemical shift range of the ^{113}Cd ion. The shift scale is relative to $\text{Cd}(\text{ClO}_4)_2$ at neutral pH.

$k_{\text{OFF}} < 50 \text{ s}^{-1}$, which corresponds to association constants $K_a > 10^6 \text{ M}^{-1}$.

For sites of lower affinity, intermediate and fast exchange conditions apply. In the case of fast chemical exchange ($k_{\text{OFF}} \gg \delta\omega$) the observed signal is narrow with a chemical shift which is a population weighted average [4]. When the exchange rate is the same order of magnitude as the chemical shift difference the NMR signals of both sites broaden, sometimes beyond detection.

3.4. Experimental Aspects

The low sensitivity of most of the nuclei discussed in this thesis brings a need for an increased signal to noise ratio. For certain nuclei such as ^{43}Ca , ^{25}Mg and ^{113}Cd this can be obtained by the use of isotopically enriched chemicals. For ^{25}Mg and ^{113}Cd the cost involved causes no problems but for ^{43}Ca a considerable investment is needed and the ^{43}Ca must generally be recovered.

The use of higher magnetic fields also increases the signal to noise ratio. When going from iron core electromagnets to cryomagnets a large portion of the sensitivity gained is lost when using the saddle-shaped

Helmholtz coils, Figure 5. This type of coil design allows the use of standard NMR tubes.

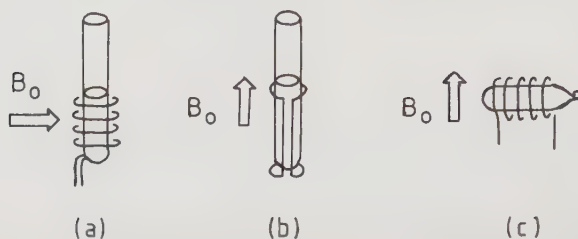


Figure 5. The coil arrangement in: a) iron core electromagnets, b) cryomagnets equipped with Helmholtz coils and c) cryomagnets equipped with solenoids.

In our laboratory we have for several years used a probe, constructed by Hans Lilja, with a horizontal sample orientation so that the higher performance solenoid coil design could be used, Figure 5c. With this procedure the signal to noise ratio was found to increase by a factor of ~ 2.5 which corresponds to a gain in time by a factor of ~ 6 .

The handling of the sample with this probe construction is more cumbersome since the solution has to be filled in small flasks of approximately 2.5 ml volume. Figure 6 shows this type of flask next to the "head" of the probe. The probe is of the broad-band type, tunable over 10-100 MHz. The sample holder is non-spinning and can be thermostated between -10 and $+100^{\circ}\text{C}$.

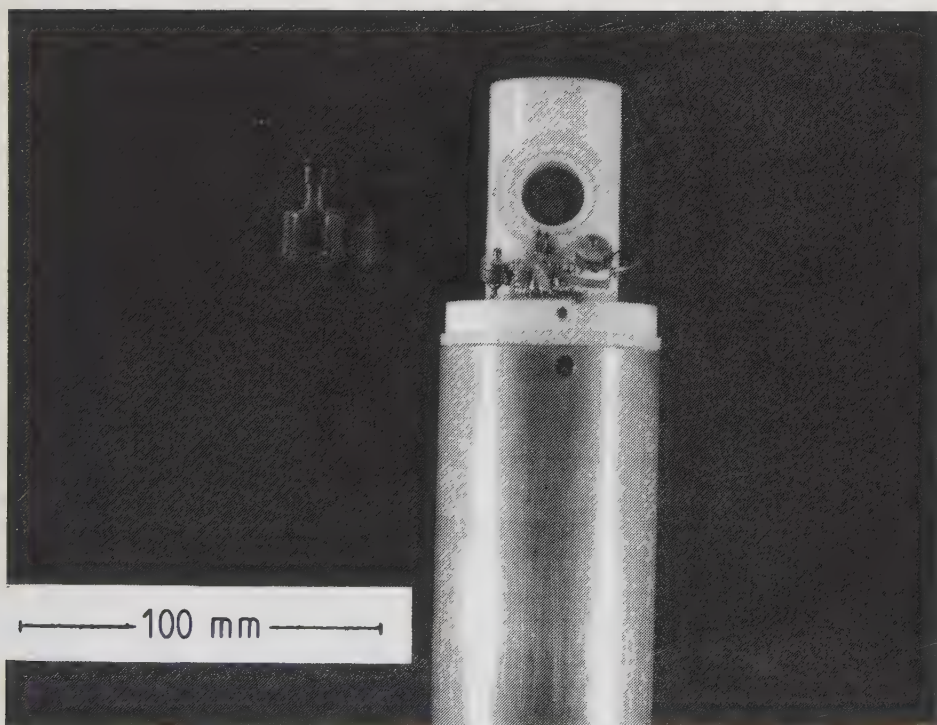


Figure 6. The head of the broad-band probe used in the ^{43}Ca , ^{25}Mg and ^{113}Cd measurements described in this thesis.

4. ANION BINDING TO HORSE HEART CYTOCHROME c

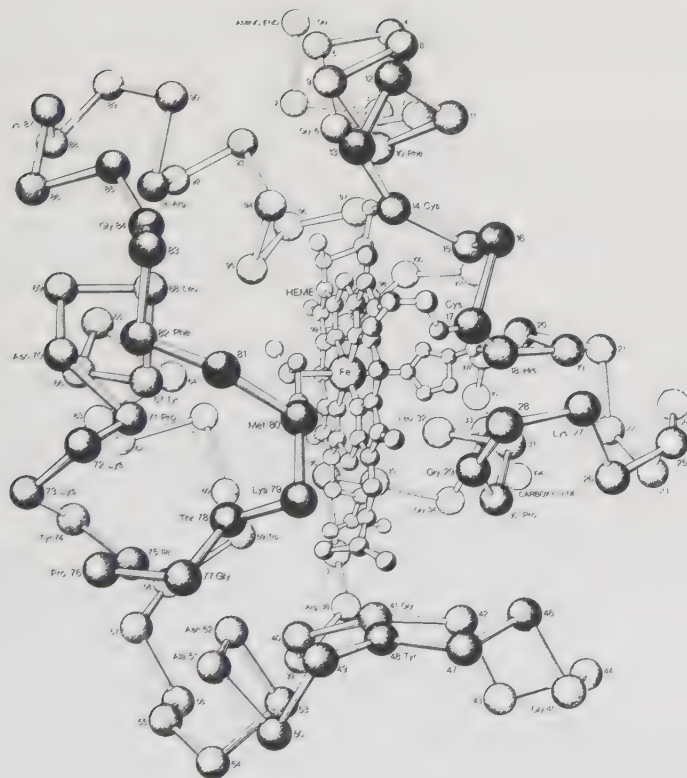


Figure 7. The peptide chain folding of tuna cytochrome c. From [13].

4.1. Introduction

Cytochrome c is a component of the biochemical respiratory chain of oxidative phosphorylation which produces ATP by transfer of electrons and protons through a series of steps ending with reduction of molecular oxygen to water [14]. Unlike most other members of the respiratory chain cytochrome c is water soluble and easily extracted from disintegrated mitochondria. For this reason cytochrome c was the first cytochrome to be studied extensively and has received most attention from biochemists.

Eucaryotic cytochrome c is a small protein composed of 103-113 amino acid residues and an iron porphyrin group covalently attached. X-ray structures have been determined by Dickerson and co-workers [15-20]. The heme group is held in a hydrophobic crevice by covalent bonds to Cys 14 and Cys 17.

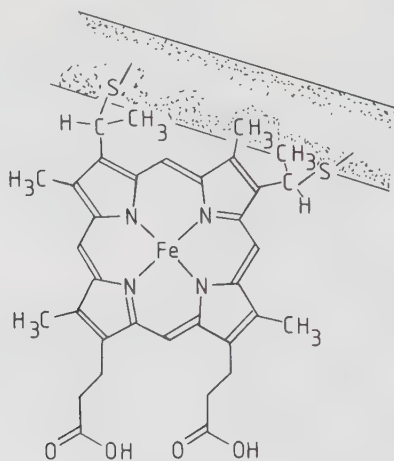


Figure 8. Heme c.

The axial ligands of the heme iron are provided by the δ -nitrogen of His 26 and the sulphur of Met 80. The heme is not completely buried by the folds of the polypeptide chain and one edge of the porphyrin ring system is in contact with the surrounding solution. This area of the protein surface is usually termed the "front side" of cytochrome c, (see Figure 7). Cytochrome c is a heavily charged protein with approximately 20 basic and 10 acidic residues. The distribution of ionic charges over the surface of the molecule is not uniform. There is a large excess of positively charged side chains on the "front side", Figure 9.

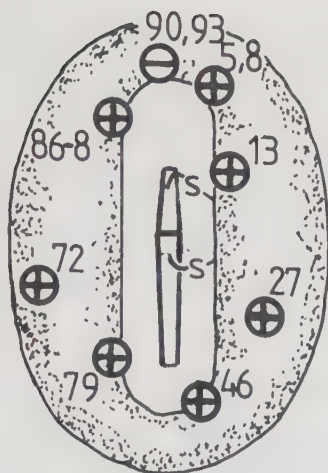


Figure 9. The positively charged residues around the exposed heme edge of cytochrome c.

The biochemical role of cytochrome c is to accept electrons from cytochrome c reductase and in turn to pass them on to cytochrome c oxidase. The available data suggests that cytochrome c forms a complex with its membrane bound oxido-reductases. The interaction is thought to be partly electrostatic, and to involve the positively charged "front" surface of cytochrome c and negatively charged domains of the oxidase and reductase [21].

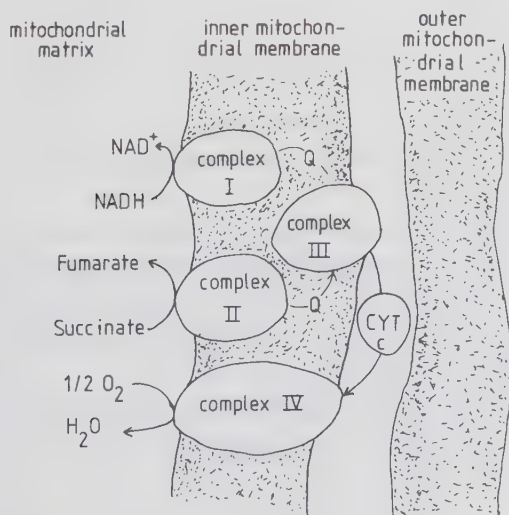


Figure 10. The role of cytochrome c as an electron-carrying shuttle.

The crystallographic structure of tuna cytochrome c has been determined for both oxidation states [15-20]. No significant differences were observed. However there is extensive physical and chemical data indicating that the physical properties of cytochrome c differ in oxidised and reduced states [21]. On the whole, the native conformation of reduced cytochrome c appears to be much more stable than the oxidised conformation. For example, reduced cytochrome c maintains a well-ordered structure over the pH range 3.5 to 12.5 and over the temperature range 4°C to 60°C [13,22].

It is known from spectroscopic studies that oxidised cytochrome c exists in five pH dependent conformational states [23]. The physiologically active one, usually denoted state III, is stable between pH 2.5 and 9.5. Above pH 9.5 it undergoes a conformational transition to state IV. This process is often referred to as the alkaline isomerisation of cytochrome c [13]. The transition is believed to be associated with the loss of Met 80 as the sixth axial heme ligand. However, another strong field ligand must coordinate to the heme iron since it remains in a low-spin state [24]. The new ligand appears to be an unprotonated amino group of lysine, either Lys 72 or Lys 79 [25-29], although this assignment is not conclusive [30]. The alkaline isomerization is readily followed by the absorption band at 695 nm. This weak band is due to a charge transfer from the sulphur of Met 80 and to the heme iron and is a sensitive indicator of the native protein [13].

The presence of polyanions and of simple anions such as Cl^- and phosphate has long been known to affect the oxidation reduction reactions of cytochrome c [31-34]. The current interpretation of this phenomenon is that anions bind to sites at the "front side" of cytochrome c and thereby hinder interaction between cytochrome c and its redox partners.

Ion binding to cytochrome c has been widely investigated. A variety of techniques have been employed including electrophoreses, redox potential measurements, gel filtration, reaction kinetics, measurements of reaction yields, ion exchange chromatography and NMR. The earliest ion binding studies on cytochrome c were made by Margoliash and co-workers using an electrophoretic technique. They came to the conclusion that oxidised cytochrome c binds Cl^- and phosphate while the reduced protein

Table 2. The conformational states of cytochrome c (from ref. 13).

A. Oxidised cytochrome c

State:	I	II	III	IV	V
pK _a of transition:	0.4	2.5	9.1	12.5	
Electron spin of iron:	High	High	Low	Low	Low
695 nm band:	no	no	yes	no	no
5th ligand:	H ₂ O	H ₂ O	His 18	His 18	His 18?
6th ligand:	H ₂ O	H ₂ O	Met 80	Lys x	OH ⁻ ?
Protein conformation:	Denatured	Unfolded	Folded	Folded	Unfolded

B. Reduced cytochrome c

State:	I	II	III
pK _a of transition:	3.5	12.5	
6th ligand:	H ₂ O	Met 80	Met 80?
Protein conformation:	Unfolded	Folded	Unfolded

binds only phosphate. Simple cations such as Na⁺, K⁺, Mg²⁺ and Ca²⁺ were found to bind only to reduced cytochrome c [35,36]. Ion binding to cytochrome c has also been studied by Schejter and co-workers using redox potentiometric and gel filtration techniques [37-40]. The conclusion drawn from these experiments is that anions bind to oxidised cytochrome c while cations bind only to the reduced protein.

The first direct observation of anion binding to cytochrome c was made by Stellwagen and Shulman on the reduced protein [41]. They estimated an association constant of $4 \cdot 10^2 \text{ M}^{-1}$ for phosphate binding (neutral pH) to a site in the vicinity of His 26. Ion binding to oxidised cytochrome c has since then been studied by observing the effect of various ions on the yield of chemical modifications of the protein [42, 43] and on the chromatographic properties of modified cytochrome c [44].

The results of the ion binding studies quoted above are partially con-

tradictory and suggested that ion binding to cytochrome c required further investigation.

4.2. Results and Discussion

The concentration dependence of the $^{35}\text{Cl}^-$ transverse relaxation rate in Figure 1, Article I, clearly demonstrates that both oxidised and reduced horse heart cytochrome c (cyt c) bind Cl^- ions. No signs of Na^+ binding to either oxidised or reduced cyt c could be detected using ^{23}Na NMR. These findings are at variance with those of Margoliash and co-workers [35, 36] and of Schejter and co-workers [37-40].

Analysis of the data obtained here shows that there are different classes of ion binding sites present and that oxidised and reduced cyt c differ significantly in the ion binding properties of the different classes. The effects of other more complex ions such as phosphate, perchlorate and iron hexacyanides show that these ions compete with chloride for common site(s). The magnitude of the decrease of the relaxation rate in the competition experiments may be taken to indicate that they compete for the "strong" class of Cl^- site(s). The fact that the relaxation rate reaches a constant non-zero level when the competing ions are in large excess indicates that there are sites that bind Cl^- much more strongly than these other ions.

The pH dependence of the transverse ^{35}Cl relaxation rates of the Cl^- and ClO_4^- ions indicates that ϵ -amino groups in lysyl side chains are responsible to a large extent for the anion binding. The "hump" in the relaxation rate for oxidised cyt c is due to increased binding of anions to the alkaline conformation, state IV. The presence of certain ions such as perchlorate (Article II) and thiocyanate (unpublished results) also appears to stabilise the alkaline conformation (state IV).

Cyanide is known to replace Met 80 as the sixth heme ligand in oxidised cyt c, thereby producing a minor conformational change [45]. Cyanide was found to greatly reduce the ^{35}Cl relaxation rate of Cl^- and ClO_4^- ions at alkaline as well as neutral pH values. The magnitude of the decrease at a $[\text{CN}^-]/[\text{cyt c}]$ ratio of 1 indicates that cyanide binding

reduces the binding only at certain sites - possibly those of the "strong" class.

Iron hexacyanides are known to change the oxidation state of cyt c. To account for the electron transfer [46] the iron hexacyanide binding site is most likely to be found near the exposed heme edge. Iron hexacyanides compete with Cl^- for the same site. We therefore suggest that this site (or sites) is located on the "front side" of cyt c. In the alkaline conformation (state IV) the structure is slightly changed resulting in an increased binding of anions to this form.

The fact that the native states of oxidised and reduced cyt c show marked differences in Cl^- affinities to the "strong" site(s) indicates that the arrangement of the surface charges around the exposed heme edge is not the same in the two redox forms.

4.3. Summary

Both oxidised and reduced cyt c have several positively charged lysyl side chains at the "front surface". It is therefore not surprising to find that both oxidation states bind small anions. There is extensive data suggesting that these ions are bound in the vicinity of the "exposed heme edge". The ion binding properties of these sites are shown to be affected under conditions known to produce small structural changes at the "front side".

The finding that oxidised and reduced cyt c show different ion binding properties indicates that the structure of the protein, in particular the surface region, is somewhat different in the two forms.

5. CATION BINDING TO PROTEINS

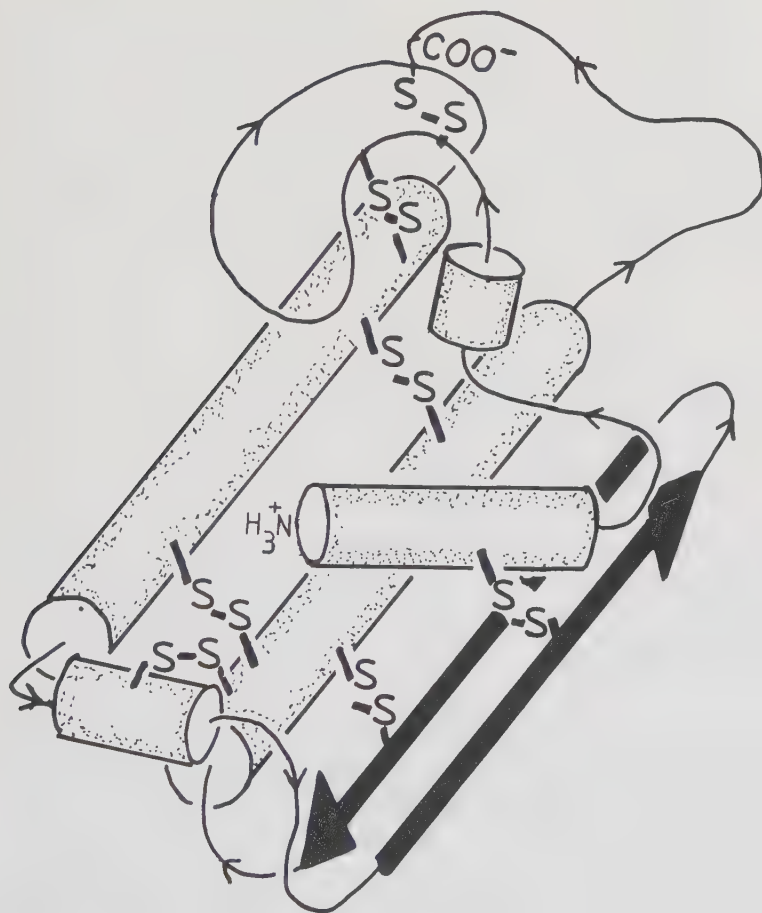
5.1. Ca^{2+} BINDING TO PORCINE PANCREATIC PHOSPHOLIPASE A_2 

Figure 11. A schematic drawing showing the α -helices and the β -structures of the bovine phospholipase A_2 molecule (after ref. 56).

5.1.1. Introduction

Phospholipase A_2 catalyses the hydrolysis of the 2-acyl linkage of phospholipids [47,48].

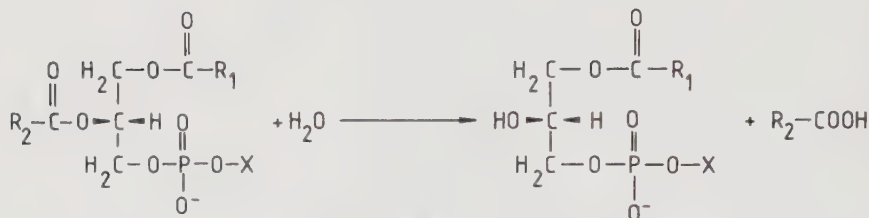


Figure 12. The hydrolysis of the 2-acyl bond of 1,2-diacyl sn-phospholipids which is catalysed by phospholipase A_2 . R_1 and R_2 are alkyl chains; X represents choline, ethanolamine, glycerol or serine.

The enzyme has been isolated from a number of sources including snake venom [49,50] bee venom [51] and from mammalian pancreas [48,52]. There are indications of the presence of an intracellular phospholipase in some mammalian tissues [53].

It has been shown that Ca^{2+} ions are essential for the catalytic properties of phospholipase. The pancreatic enzymes are secreted by the pancreas in an inactive form. In the duodenum the zymogens are activated by tryptic cleavage. The pancreatic enzymes are relatively small macromolecules consisting of a single polypeptide chain of 123-125 amino acid residues ($M_w \sim 14 \cdot 10^3$ D); the zymogen possesses an additional 7 residues at the N-terminus [54].

The crystal structure of bovine phospholipase A_2 has been determined by Dijkstra et al. [55,56]. The molecule has the shape of a flattened cylinder of approximately $22\text{\AA} \times 30\text{\AA} \times 42\text{\AA}$. The protein has a rather high content of secondary structure (50% α -helix and 10% of β -sheet). The enzyme also contains 7 disulphide bridges which hold the protein firmly

together (Figure 11). At the time of writing there is no available crystallographic structure for the proenzyme.

Both the active enzyme and the zymogen have been shown to bind Ca^{2+} in a 1:1 molar ratio with association constants in the range of $1\text{--}5 \cdot 10^3 \text{ M}^{-1}$. Evidence has been presented for an additional cation binding site in the active porcine enzyme, however with a much lower affinity: $K_{\text{Ca}}^w \sim 50 \text{ M}^{-1}$ [57]. In bovine phospholipase A_2 the strong Ca^{2+} binding site is located in the active site with coordination by 7 oxygen ligands (Figure 13). Ba^{2+} and Sr^{2+} ions bind to this site with association constants comparable to that of Ca^{2+} but the enzyme loses its activity. No indications of Mg^{2+} binding to the active site have been observed [58].

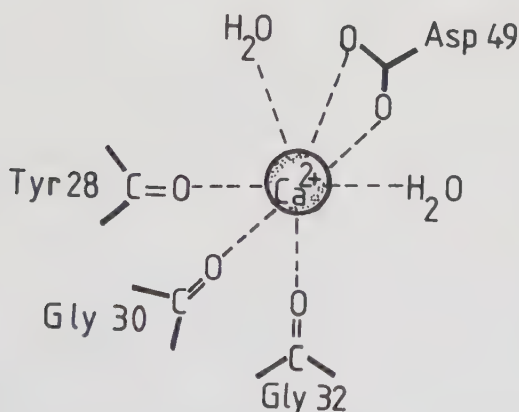


Figure 13. A schematic representation of the coordination of the active site Ca^{2+} ion in bovine phospholipase A_2 (from ref. 55).

The amino acid residues that form the active site, judging from the phospholipase sequences known so far, are highly invariant. This indicates that the structure of the active site is the same in molecules from all sources [56]. A hypothesis for the role of the Ca^{2+} ion in the catalytic process has been developed by Verheij et al. [59].

Several investigations have suggested that there is an active site in prophospholipase A_2 similar to the one in the active enzyme. The zymogen is capable of degrading monomeric substrate molecules. On tryptic

cleavage the protein undergoes a minor conformational change resulting in the formation of the "interfacial recognition site" (IRS) which enables the enzyme to associate with aggregated substrate molecules. The formation of the enzyme-aggregate complex increases the turnover of substrate molecules dramatically [60]. The α -amino group of Ala 1 has been shown to play an essential role in the formation of the IRS [54,61].

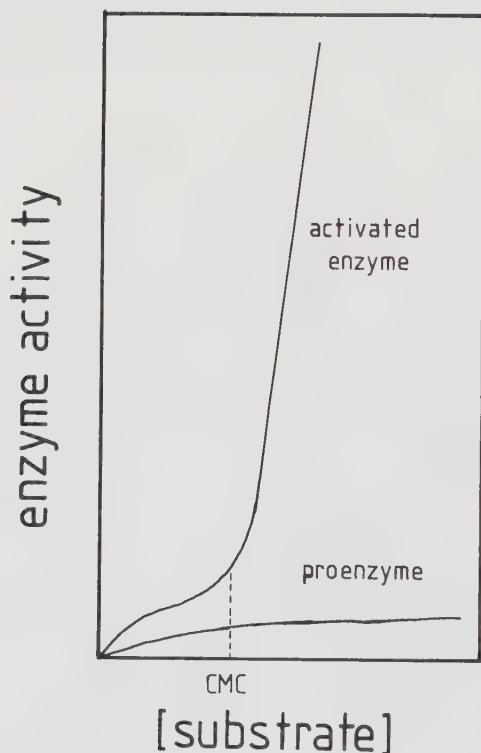


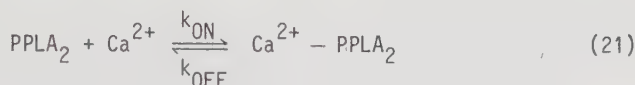
Figure 14. The substrate concentration dependence of the activity of phospholipase A_2 and phospholipase A_2 (from ref. 60). CMC denotes the concentration above which substrate molecules begin to form micelles (short fatty acid chains in the phospholipid).

The aim of this investigation was to study the ion binding properties of both the active form and the pro-form of phospholipase A₂ with special emphasis on the kinetics of the Ca²⁺ binding to the active site.

5.1.2. Ca²⁺ Binding to the Zymogen

Ca²⁺ binding to the pro-form of porcine pancreatic phospholipase A₂ (PPLA₂) is dealt with in Article III.

In the presence of PPLA₂ the ⁴³Ca NMR signal of a CaCl₂ solution at neutral pH is significantly broadened due to the binding of Ca²⁺ ions to the active site. The data in Figure 1 in Article III can be fitted to a model with a simple chemical equilibrium:



This gave an association constant $K^{\text{PRO}} = 2.5 \pm 1 \cdot 10^3 \text{ M}^{-1}$ at 24°C, (Table 3).

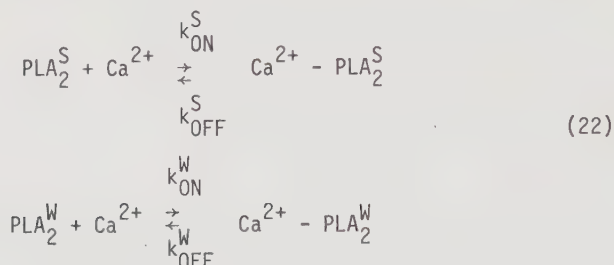
The temperature dependence of the ⁴³Ca NMR line width (Figure 2 in Article III) is a textbook example of a "slow-exchange" – "fast-exchange" transition. As described in the theoretical section the data can be analysed to obtain an estimate of the rate of exchange of Ca²⁺ between the active site and the bulk solution: $k_{\text{OFF}} = 3.0 \pm 0.5 \cdot 10^3 \text{ s}^{-1}$ at 24°C, Table 3 (New value).

The pH dependence of the ⁴³Ca line width (Figure 3 in Article III) can be used to obtain an apparent pK value for the Ca²⁺ binding group(s). Fitting a model with a single protonation step gives $\text{pK}_a = 5.2$. This value most likely represents the ionization of Asp 49, the only charged ligand of the Ca²⁺ ion on the active site [55].

5.1.3. Ca²⁺ Binding to the Active Enzyme

Ca²⁺ binding to porcine pancreatic phospholipase A₂ (PLA₂) is described in this section. Figure 15 shows the ⁴³Ca line width for a PLA₂ solution,

neutral pH, at varying Ca^{2+} concentrations. A closer look at the data shows that the simple model employed for the pro-enzyme is not sufficient to describe the data. A model with two independent sites, one strong site - the active site - and one weak site, was however satisfactory.



The strong site ($K^S \approx 1 \cdot 10^3 \text{ M}^{-1}$) describes the binding constant of Ca^{2+} ions to the active site. The weak site ($K^W \approx 15 \text{ M}^{-1}$) is not found in the zymogen and has consequently been formed during the activation process.

The presence of two classes of Ca^{2+} binding site is further demonstrated in the pH dependence of the ^{43}Ca line width. Figure 16a shows the pH dependence at a $[\text{Ca}^{2+}]/[\text{PLA}_2]$ ratio of ~ 1 . To obtain a good fit a two-site model with the apparent pK values 5.7 and 4.6 was used. The pK_a value of 5.7 accounts for 87 % of the total change in the line width and most likely describes the titration of Asp 49, the charged ligand at the Ca^{2+} ion in the active site. Figure 16b shows the same experiment at a tenfold higher $[\text{Ca}^{2+}]/[\text{PLA}_2]$ ratio. The relative step associated with the pK_a value is ~ 5.7 is now reduced to 30 %. This is due simply to changes in the fraction of ions bound to the active site and to the "weak" site.

Figure 17 shows the temperature dependence of the ^{43}Ca signal for a CaCl_2 solution containing PLA_2 (neutral pH). The data was fitted to the model of Equation 22. The "weak" site was assumed to bind Ca^{2+} ions so that fast exchange conditions applied throughout the temp. range studied. The exchange rate of the active site Ca^{2+} ion was calculated to be: $k_{\text{OFF}}^S = 1.0 \pm 0.1 \cdot 10^3 \text{ s}^{-1}$, Table 3.

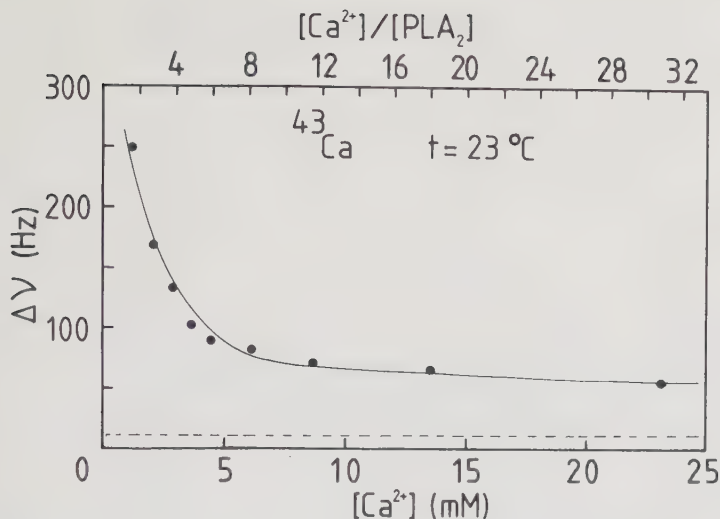


Figure 15. The ^{43}Ca NMR signal of a 0.74 mM PLA_2 solution, pH 7.1, at different Ca^{2+} concentrations, recorded at 23°C .

5.1.4. Conclusions

The Ca^{2+} binding properties of phospholipase A_2 undergo a slight change on tryptic activation. This is apparently due to a conformational change affecting the geometry of the active site that is shown by an increase in the quadrupole coupling constant, a decrease in the Ca^{2+} affinity and a change in the apparent pK value of Asp 49 by ~ 0.5 pH units, Table 3.

The values of the calculated on-rates given in Table 3 are approximately two orders of magnitude smaller than if this rate was diffusion controlled [62] indicating that the on-processes are sterically hindered. Again we can detect a significant difference between the two forms - the on-rate is approximately six times slower for the active enzyme. This indicates that the Ca^{2+} binding site is more "shielded" in the active enzyme.

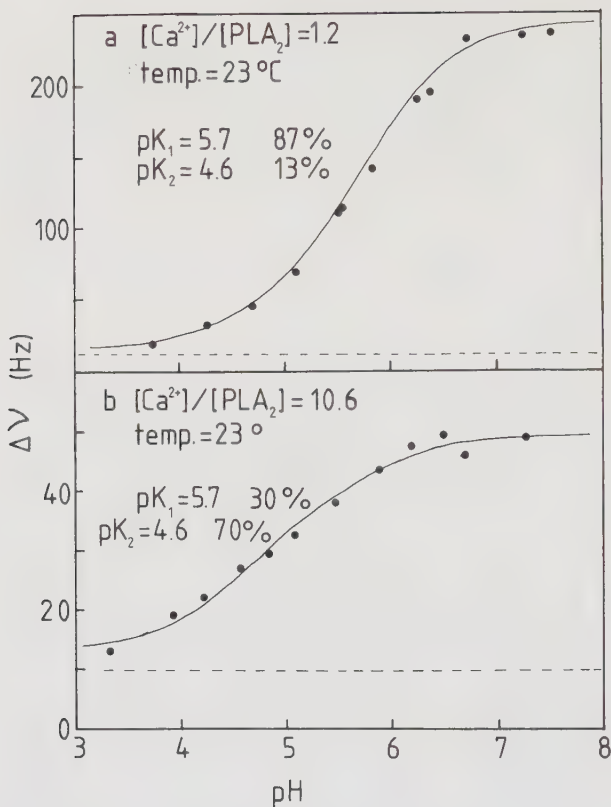


Figure 16. The pH dependence of the ^{43}Ca NMR line width for a) a 1.1 mM CaCl_2 solution containing 0.98 mM PLA_2 and b) a 11.5 mM CaCl_2 solution containing 1.1 mM PLA_2 . The data was recorded at 23°C.

The formation of a second "weak" Ca^{2+} binding site has earlier been reported by Slotboom et al. [57]. Our value of the binding constant $K^W = 15 \text{ M}^{-1}$ is in fair agreement with the value reported by these authors ($K = 50 \text{ M}^{-1}$). The biochemical significance of this state has not been demonstrated. The magnitudes of the binding constant and the pK_a values suggest that this site is located at the surface of the active protein (similar to the weak sites of hemocyanin, cf. section 5.4).

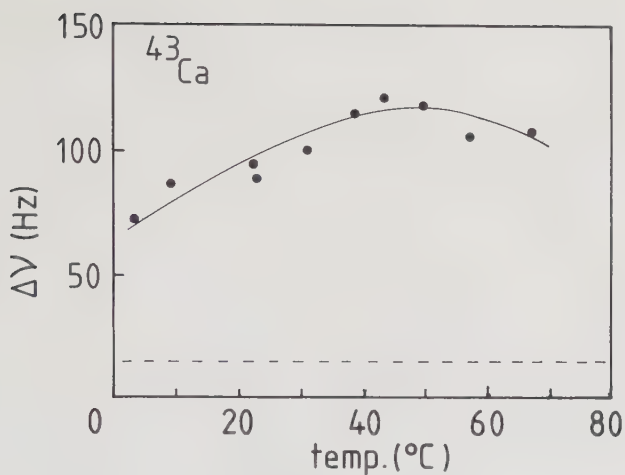


Figure 17. The temperature dependence of the ^{43}Ca NMR line width for a 4.4 mM CaCl_2 solution, pH 7.2, containing 0.74 mM PLA_2 .

Table 3. The NMR and Ca^{2+} binding parameters obtained for PPLA_2 and PLA_2 :

	PPLA_2	PLA_2	
	ACTIVE SITE	ACTIVE SITE	WEAK SITE
K (M^{-1})	2500 ± 1000	1000 ± 200	15
pK_a	5.2	5.7	4.6
X (MHz)	0.8	1.5	-
k_{OFF} (s^{-1})	3000 ± 500^b	1300 ± 300	-
k_{ON} ($\text{s}^{-1}\text{M}^{-1}$) ^a	$7.5 \cdot 10^6$	$1.3 \cdot 10^6$	-

^a Calculated from the relation: $k_{\text{ON}}/k_{\text{OFF}} = K$.

^b New calculation of the data in Article III.

5.2. Ca^{2+} AND Mg^{2+} BINDING TO RABBIT SKELETAL MUSCLE TROPONIN C

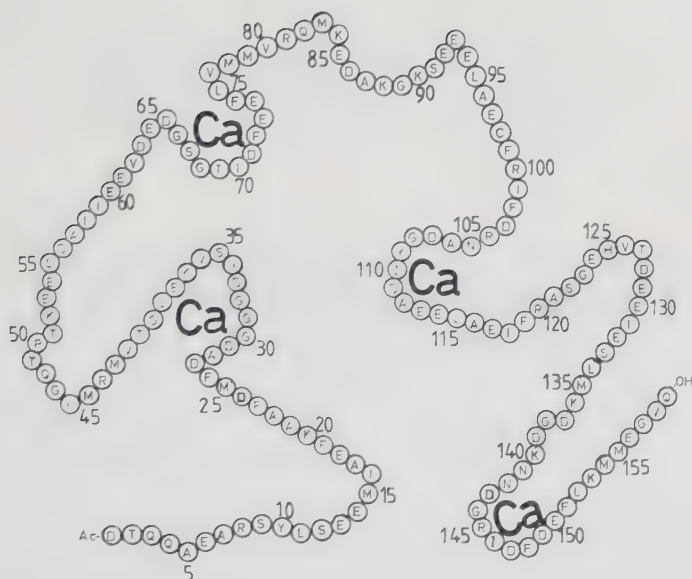


Figure 18. The amino acid sequence of rabbit skeletal muscle troponin C, (A-Ala, R-Arg, N-Asn, D-Asp, C-Cys, Q-Gln, E-Glu, G-Gly, H-His, I-Ile, L-Leu, K-Lys, M-Met, F-Phe, P-Pro, S-Ser, T-Thr, W-Trp, Y-Tyr, V-Val). The calcium binding sites are tentatively assigned from sequence analogies with carp parvalbumin $pI = 4.25$ [63].

5.2.1. Introduction

The contractile cells of skeletal muscle are extremely long. Their plasma membrane is known as the sarcolemma. The contractile elements of muscle cells are the myofibrils which are arranged in parallel bundles along the axis of contraction. Muscle cells also contain a highly differentiated endoplasmic reticulum called the sarcoplasmic reticulum. Myofibrils show a structural pattern that repeats every $2.5\ \mu\text{m}$. These repeated units, called sarcomeres, are composed of two types of filaments, the thin and thick filaments [64-67].

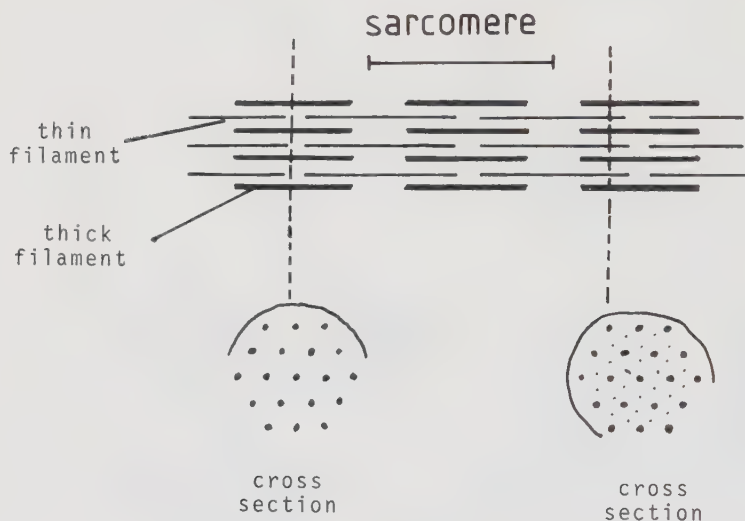


Figure 19. The sarcomere of skeletal muscles [14].

The thick filaments consist mainly of myosin molecules. Myosin is a large protein, $M_w \sim 4.6 \cdot 10^5$ D, which is an ATPase. Myosin is built up of two identical major chains with a rod-like 1340 Å long α -helical region and a globular region of approximately 90 Å diameter [14].

The major constituent of the thin filaments is actin, a $M_w \sim 4.2 \cdot 10^4$ D protein, which under physiological conditions polymerises into a double-stranded helix. Other important components of the thin filament are tropomyosin and the troponin complex. Tropomyosin is a double-stranded

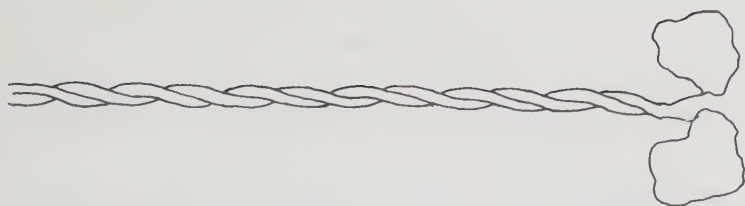


Figure 20. The myosin molecule.

helical rod of ~ 400 Å length, and is located in the groove between two helical actin strands [14]. Troponin is a complex of the three subunits troponin I ($M_w \sim 2.4 \cdot 10^4$ D), troponin C ($M_w \sim 1.8 \cdot 10^4$ D) and troponin T ($M_w \sim 3.7 \cdot 10^4$ D). The troponin complex is located in the thin filaments at intervals of ~ 400 Å.

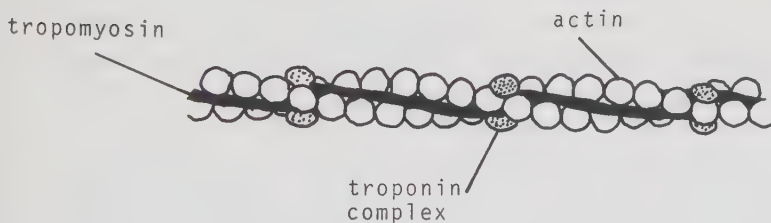


Figure 21. Model suggested for thin filaments [14].

Several investigations have suggested that the force of muscular contraction arises from an interaction between myosin and actin [14]. The regulation of muscle contraction involves the binding of Ca^{2+} ions to the troponin complex. In the relaxed state, the Ca^{2+} is stored in the sarcoplasmic reticulum. An excitation of the muscle cell causes a release of Ca^{2+} from the sacs of the sarcoplasmic reticulum. The initial event in the contraction process is generally considered to be the binding of Ca^{2+} to the troponin complex which then undergoes a conformational change. In a series of events that are less well documented this conformational change results in the

sliding of the thin and thick filaments past each other [14]. The Ca^{2+} binding component of the troponin complex is troponin C.

5.2.2. Troponin C

Troponin C, calmodulin and parvalbumin form a group of Ca^{2+} binding proteins of similar amino acid composition with isoelectric points around 4.5. The crystal structure of carp parvalbumin ($\text{pI} = 4.25$) is known from x-ray studies by Kretsingers group [68]. Parvalbumins bind two Ca^{2+} ions with relatively high affinity ($K_a \sim 10^7\text{-}10^9 \text{ M}^{-1}$), [69-70] at sites formed in a kink between two α -helical segments [68]. In this type of site the Ca^{2+} ion is octahedrally coordinated by oxygen atoms from the amino acid side chains and the peptide chain. The sites described above have also been shown to bind Mg^{2+} ions, though with lower affinity ($K_a \sim 10^5 \text{ M}^{-1}$, [70]).

To date no crystal structure of troponin C has been presented. However the amino acid sequence of rabbit skeletal muscle troponin C (TnC) has been determined by Collins et al. [63]. Comparison with the primary structure of carp parvalbumin ($\text{pI} = 4.25$) indicates the presence of four Ca^{2+} binding sites [63]; see Figure 18.

Cation binding to TnC has been studied by Potter and Gergely [71] using an equilibrium dialysis technique. TnC was shown to have two Ca^{2+} binding sites with association constants K_a around $2 \cdot 10^7 \text{ M}^{-1}$ (the "high affinity" sites) and two sites with a somewhat lower affinity $K_a \sim 3 \cdot 10^5 \text{ M}^{-1}$ (the "regulatory" sites). Mg^{2+} ions are reported to bind to the "high affinity" sites with an association constant of $\sim 5 \cdot 10^3 \text{ M}^{-1}$ [71]. Potter and Gergely state that the "regulatory" sites are Ca^{2+} specific, i.e. that under the experimental conditions used no Mg^{2+} binding could be detected.

The binding of Ca^{2+} and Mg^{2+} to the cation binding sites of TnC described above is known to produce substantial changes in protein folding. Hincke et al. [72] followed the circular dichroism (CD) of TnC as a function of added Ca^{2+} . The results clearly demonstrated the presence of different classes of sites with binding constants

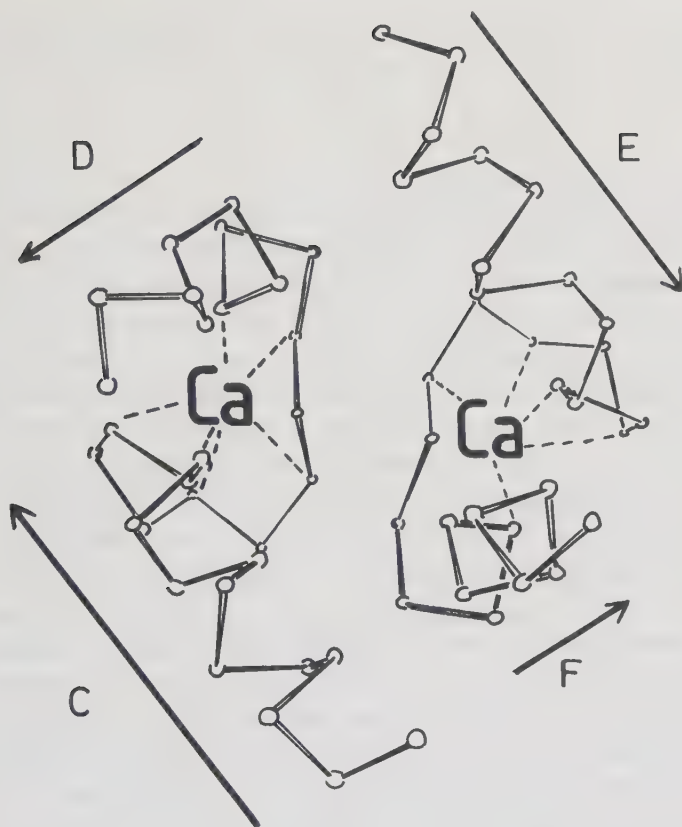


Figure 22. Schematic representation of the α -carbons of the peptide chain around the Ca^{2+} binding sites in carp parvalbumin (pI = 4.25) from ref. 7.

similar to those of Potter and Gergely [71]. From the CD changes associated with each class of site it is apparent that Ca^{2+} binding to the "high affinity" sites elicits a larger conformational change (ca. three times greater) than that associated with Ca^{2+} binding to the "regulatory" sites. This is also evident from ^1H NMR studies by Seamon et al. [73] and Levine et al. [74,75]. These studies indicate that Mg^{2+} binding to the "high affinity" sites leads to changes in the ^1H NMR spectrum similar to those found when Ca^{2+} is bound to these sites. This finding suggests that the conformation of TnC remains about the same when Ca^{2+} ions are replaced by Mg^{2+} ions at the "high affinity" sites.

There is also evidence for a third class of sites which bind Mg^{2+} and Ca^{2+} ions with association constants around 10^3 M^{-1} (the "weak" sites) [71,72].

When TnC is associated with the other members of the troponin complex the Ca^{2+} affinities for all four sites are an order of magnitude higher than for the TnC molecule alone [71]. In the relaxed muscle cell $[\text{Ca}^{2+}] \approx 10^{-8} \text{ M}$ and $[\text{Mg}^{2+}] \approx 10^{-3} \text{ M}$ [76] the "high affinity" sites are populated by Mg^{2+} ions according to these binding constants. When the Ca^{2+} concentration is increased to about 10^{-5} M on excitation, the Mg^{2+} ions bound to the "high affinity" sites are replaced by Ca^{2+} and at the same time Ca^{2+} ions are bound to the "regulatory" sites. The prevailing picture of the roles played by the two divalent cations in the triggering process is as follows: Ca^{2+} and Mg^{2+} bound to the "high affinity" sites serve as "structural" elements maintaining the protein folding while the Ca^{2+} ions bound to the "regulatory" sites are believed to be involved in triggering the muscular contraction [76]. The contraction and relaxation of skeletal muscle is a dynamic process enacted on a millisecond time scale. In the assessment of the roles played by the different cation binding sites on TnC, the rates of the conformation changes produced upon binding and release of ions to these sites are significant parameters. These rates have been determined by Johnson et al. [76] by a stopped flow technique with a fluorescent probe attached to the protein. The correlation between these values and the metal ion exchange rates estimated here will be discussed in this chapter.

5.2.3. Summary of Results and Discussion

Metal Ion Titration

Ca^{2+} titration of apo-TnC: The presence of TnC in a ^{43}Ca solution at neutral pH causes significant broadening of the ^{43}Ca NMR signal. Up to a $[\text{Ca}^{2+}]/[\text{TnC}]$ ratio of two the observed ^{43}Ca line width is constant, ca. 800 Hz, and is shifted ca. 10 ppm downfield relative to the signal for "free" $^{43}\text{Ca}^{2+}$. Under the experimental conditions used only the "high affinity" sites are populated (the concentration of free, exchangeable

$^{43}\text{Ca}^{2+}$ ions is exceedingly low) and thus the observed ^{43}Ca NMR signal originates from $^{43}\text{Ca}^{2+}$ ions bound to these sites. Measurements of the longitudinal relaxation rate in combination with the transverse relaxation rate obtained from the line width at half height yield a value for the correlation time of $\tau_c \approx 11 \pm 2$ ns at 23°C (Article VI). This value probably represents the correlation time of the rotational diffusion of the entire protein molecule. Calculation from the correlation time and the transverse relaxation time gives a quadrupole coupling constant of 1.05 ± 0.05 MHz.

At higher $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios the ^{43}Ca signal is shifted upfield and the line width decreases. In this case ($[\text{Ca}^{2+}]/[\text{TnC}] > 3$) the observed ^{43}Ca signal is an average, exchange-broadened signal due to the binding of Ca^{2+} ions to the "regulatory" sites of TnC. Assuming independent sites with equal Ca^{2+} affinities this gives a lower limit for the association constant for the regulatory sites of $K_{\text{Ca}}^{\text{REG}} > 10^4 \text{ M}^{-1}$.

Mg²⁺ titration of apo-TnC. As for ^{43}Ca the ^{25}Mg signal is broadened in the presence of TnC at pH values around neutrality. The magnitude of the Mg^{2+} binding constants derived by Potter and Gergely [71] indicated that the observed ^{25}Mg signal will be an exchange-broadened signal. This seems to be the case judging from the data in Article IV, Figure 4, (it should be noted that ^{25}Mg unlike ^{43}Ca , does not allow direct observation of signals from ions tightly bound to proteins of this size since the larger ^{25}Mg quadrupole moment will result in line widths of the order of 10^4 Hz).

The observed ^{25}Mg broadening is due to the binding of Mg^{2+} ions to several classes of site. However, most of the broadening is due to Mg^{2+} binding to the "high affinity" sites of TnC (see below). A model with two independent and equally strong Mg^{2+} binding sites when fitted to the results gives an association constant $K_{\text{Mg}}^{\text{HAS}} \approx 5 \pm 1 \cdot 10^2 \text{ M}^{-1}$.

Temperature Dependence

The temperature dependence of the ^{43}Ca NMR signal at three different $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios is shown in Figure 23. The observed broadening of the ^{43}Ca signals is due mainly to Ca^{2+} ions exchanging with the "regulatory" sites (except for the lowest $[\text{Ca}^{2+}]/[\text{TnC}]$ ratio, where the observed line shape may be affected by the signal from the "high affinity" sites). As described in the theoretical section this data can be used to obtain the kinetics of metal ion binding to the "regulatory" sites. At 23°C the result of the analysis is: $k_{\text{off}} = 1.0 \pm 0.1 \cdot 10^3 \text{ s}^{-1}$, see Table 4.

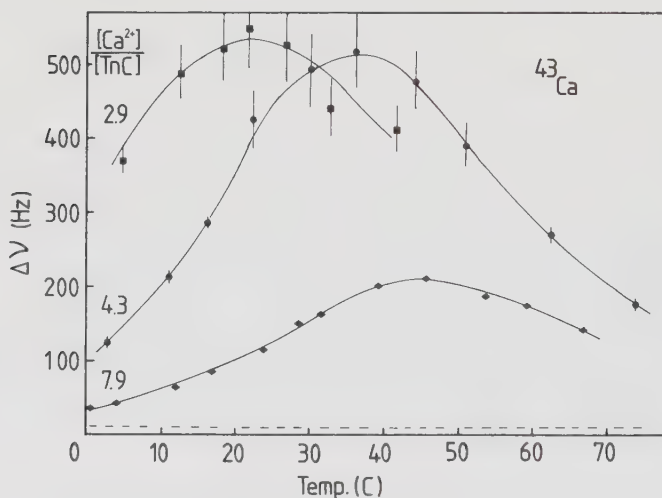


Figure 23. The temperature dependence of the ^{43}Ca line width for Ca^{2+} solutions containing TnC at neutral pH values. (■) $[\text{TnC}] = 0.56 \text{ mM}$, $[\text{Ca}^{2+}] = 1.6 \text{ mM}$; (●) $[\text{TnC}] = 0.85 \text{ mM}$, $[\text{Ca}^{2+}] = 3.7 \text{ mM}$; (◆) $[\text{TnC}] = 0.75 \text{ mM}$, $[\text{Ca}^{2+}] = 5.9 \text{ mM}$.

The temperature dependence of the ^{25}Mg NMR signal at a $[\text{Mg}^{2+}]/[\text{TnC}]$ ratio of ~ 37 is shown in Figure 24. A band shape analysis of the data gives $k_{\text{OFF}} = 8 \pm 5 \cdot 10^3 \text{ s}^{-1}$ as an average value for all of the sites observed.

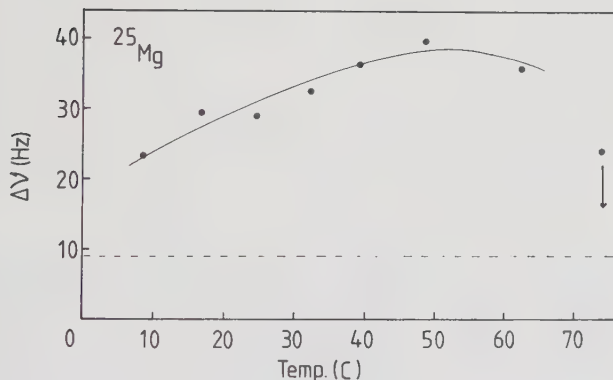


Figure 24. The temperature dependence of the ^{25}Mg NMR line width for a 7.5 mM MgCl_2 solution, pH 7.2, containing 0.2 mM TnC.

pH Dependence

The pH dependence of the ^{43}Ca NMR signal at two different $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios is shown in Figure 25. A closer look at the data shows that the decrease in the line width at acidic pH values cannot be described by a single one-protonation step. At least two pK_a values have to be introduced. A fit of this model to the relaxation data yields the apparent pK values: 4.6 - 4.9 and 6.0 - 6.5. At the $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios used, only the two weaker classes of site are probed. A simple interpretation of the presence of two classes of pK_a values is that the two "regulatory"

sites titrate at different pH values. Another explanation is that the site with the apparent pK value 6-6.5 derives from Ca^{2+} ions exchanging with the "weak" sites of TnC. At the present time we cannot rule out either of these explanations.

The pH dependence of the ^{25}Mg NMR signal is shown in Figure 26. The data can be described using a single protonation step with the apparent pK value 5.5. At the $[\text{Mg}^{2+}]/[\text{TnC}]$ ratio used the observed ^{25}Mg broadening is due to binding to both the "high affinity" and the "weak" sites, and the observed pK_a value describes the $\text{H}^+ - \text{Mg}^{2+}$ competition for both of these classes of site.

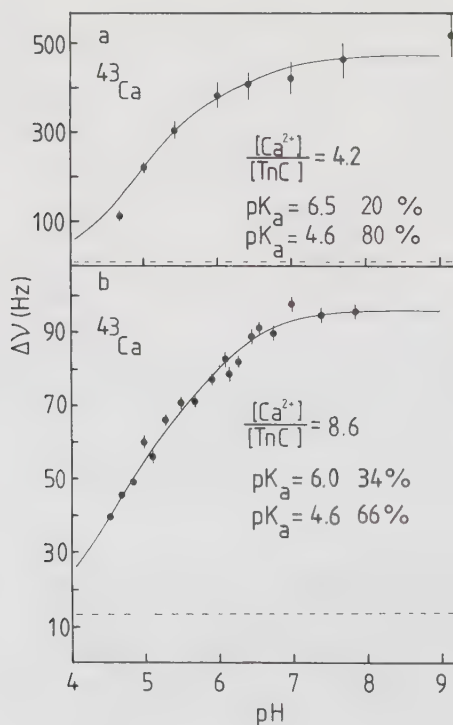


Figure 25. The pH dependence of the ^{43}Ca NMR line width for a) a 3.7 mM CaCl_2 solution containing 0.88 mM TnC and b) a 5.9 mM CaCl_2 solution containing 0.69 mM TnC. The data were recorded at 23°C .

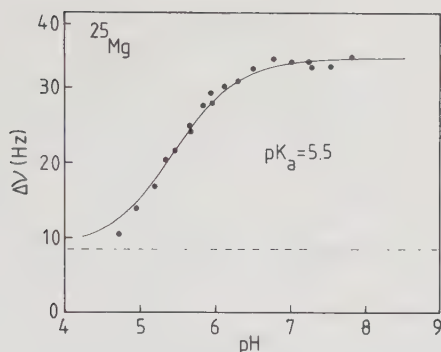


Figure 26. The pH dependence of the ^{25}Mg NMR line width for a 7.5 mM MgCl_2 solution containing 0.2 mM TnC, at 23°C.

Competition Experiments

The effect of addition of Ca^{2+} ions on the ^{25}Mg NMR line width is seen in Figure 27. The data indicate that Ca^{2+} ions displace the Mg^{2+} ions bound to the "high affinity" sites. This is in agreement with the association constants of Potter and Gergely [71]. After the addition of 2 moles Ca^{2+} per mole TnC approximately 20 % of the initial ^{25}Mg broadening remains. This is indicative of Mg^{2+} binding to sites other than the "high affinity" sites, either to the "regulatory" or to the "weak" sites or possibly both. An analysis of the data points at high Ca^{2+} concentrations shows that Ca^{2+} and Mg^{2+} ions bind to these sites with approximately the same affinity. If the remaining broadening was due to the "regulatory" sites one would expect a much steeper decrease than that seen in Figure 27 since these sites are known to bind Ca^{2+} ions much more strongly than Mg^{2+} ions. Our results suggest that the ^{25}Mg broadening at high Ca^{2+} concentrations is due to binding to the "weak" sites.

The effect of addition of Mg^{2+} on the ^{43}Ca NMR signal is seen in Figure 28. The line width is decreased by about 50 % of the initial value at high Mg^{2+} concentrations (50 moles Mg^{2+} /mole TnC). The data was recorded using a $[\text{Ca}^{2+}]/[\text{TnC}]$ ratio of 4 and under these conditions only the "high affinity" and "regulatory" sites are populated by Ca^{2+}

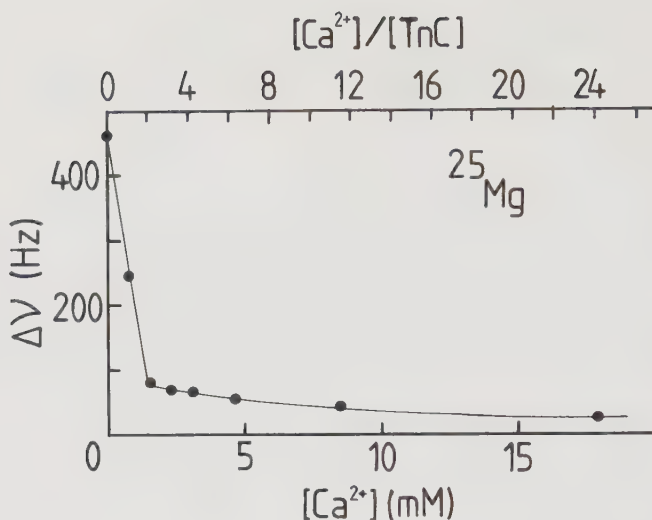


Figure 27. The dependence of the ^{25}Mg NMR line width on the concentration of CaCl_2 for a 2.9 mM MgCl_2 solution, pH 7.1, in the presence of 0.74 mM TnCl, at 23°C.

ions. It therefore appears unlikely that the decrease in the ^{43}Ca line width results from a competition between Mg^{2+} and Ca^{2+} ions for common sites.

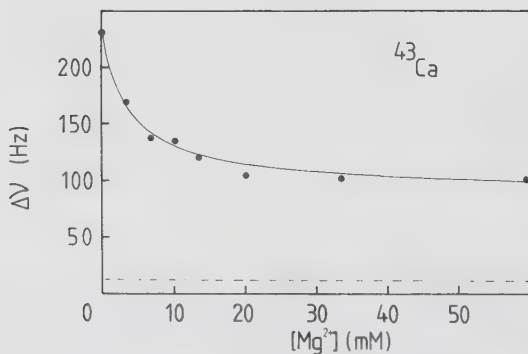


Figure 28. The effect on the ^{43}Ca NMR line width of Mg^{2+} for a 3.0 mM Ca^{2+} solution, pH 7.1, containing 0.75 mM TnCl. Temp.: 23°C.

Figure 29 shows the temperature dependence of the ^{43}Ca relaxation rate in a TnC solution in the absence of Mg^{2+} and in the presence of saturating amounts of Mg^{2+} . The temperature profile of the ^{43}Ca line width for the samples containing Mg^{2+} does not show the typical slow exchange fast exchange transition of the sample which contains only Ca^{2+} . It appears that the assumption of two identical "regulatory" sites is no longer valid. A model with two "regulatory type" sites with exchange rates differing by a factor of about 20 was found to fit the data, (Table 4).

The pH dependence of the ^{43}Ca NMR signal in the presence of excess Mg^{2+} (Figure 30), in contrast to the data in Figure 25, can be simulated with a single pK value around 4.7. This can possibly be explained by the fact that the contribution from the "regulatory" site with the slowest Ca^{2+} exchange (corresponding to $\text{pK}_a = 6.0-6.5$) is negligible at room temperature. Another explanation is that the line broadening associated with the pK_a value 6.0-6.5 derives from Ca^{2+} ions exchanging with the "weak" site(s). In the presence of large amounts of Mg^{2+} the line broadening is eliminated due to competition between Mg^{2+} and Ca^{2+} for this site(s). The resulting picture of the effect of Mg^{2+} is that it binds to a site(s) in the vicinity of one of the "regulatory" sites and thereby decreases the exchange rate of Ca^{2+} ions at this site. An approximate binding constant of $K_{\text{Mg}}^W = 5 \cdot 10^2 \text{ M}^{-1}$ can be derived from the data in Figure 28.

5.2.4. The Effect of a Phenothiazine Drug on Ca^{2+} Binding to TnC

Trifluoperazine (TFP) is an antipsychotic drug of the phenothiazine type. This class of tranquilliser has recently been considered to exert its pharmacological action via calmodulin, as will be described in the next chapter. Since calmodulin and TnC are members of the same family it is of interest to see how this drug affects TnC.

Figure 31 shows the effect of TFP on the ^{43}Ca line width for a solution containing 5.7 moles Ca^{2+} /mole TnC. The figure indicates that approximately 6 TFP molecules bind with high affinity to each TnC molecule. This result is essentially in accordance with those of Levine and Weiss from equilibrium dialysis experiments [78]. The decrease in the line width

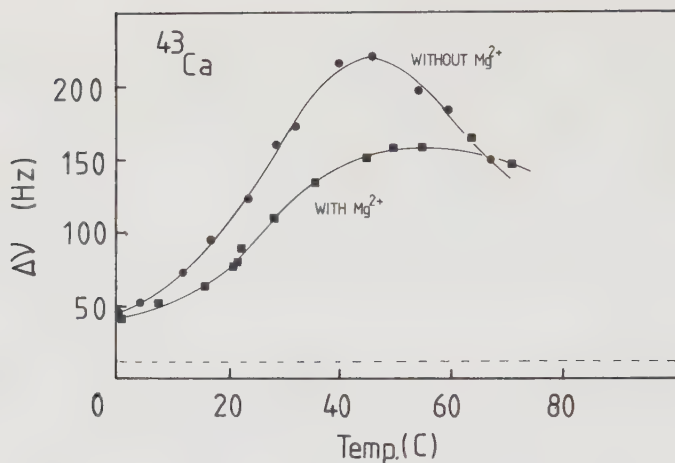


Figure 29. The temperature dependence of the ^{43}Ca line width for (●) a 5.9 mM Ca^{2+} solution in the presence of 0.75 mM TnC and (■) a 2.9 mM Ca^{2+} solution in the presence of 20 mM Mg^{2+} and 0.75 mM TnC. The data were recorded at neutral pH.

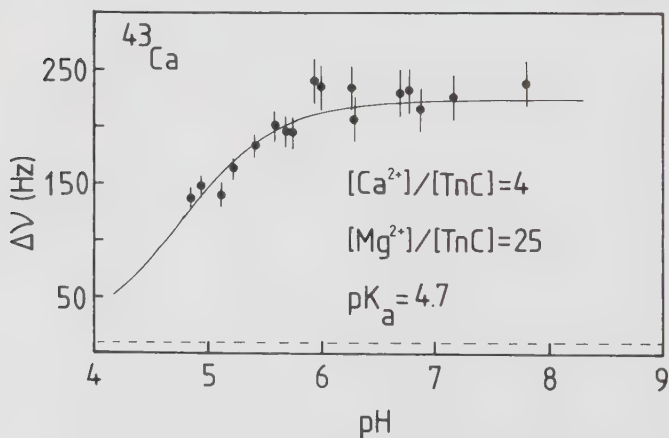


Figure 30. The pH dependence of the ^{43}Ca line width for a 3.2 mM CaCl_2 solution containing 21 mM MgCl_2 and 0.82 mM TnC, at 23°C.

can be due either to a reduction in the fraction of ions bound or to a decrease in the exchange rate. Figure 32 shows the latter explanation to be valid. An analysis of the temperature data indicates that the Ca^{2+} ion exchange rate between the "regulatory" sites and the bulk solutions is reduced by a factor of $\sim 10^2$.

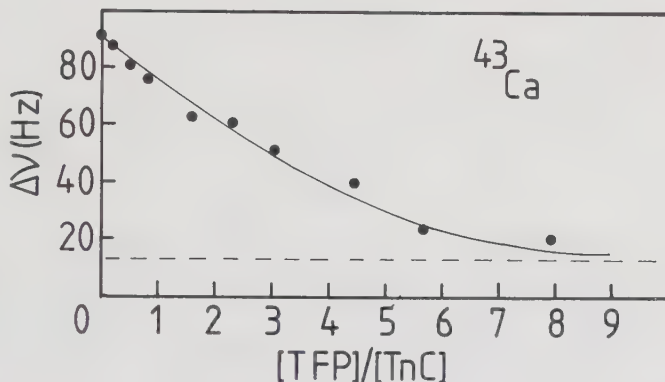


Figure 31. The dependence of the ^{43}Ca NMR line width on the concentration of TFP for a 5.9 mM CaCl_2 solution, pH 7.2, containing 1.0 mM TnC.

Figure 33 shows the ^{113}Cd NMR spectrum of TnC (4 moles of Cd^{2+} /mole TnC) at different concentrations of TFP. In the absence of TFP only the two ^{113}Cd signals from the "high affinity" sites are observed. The two ^{113}Cd signals from the "regulatory" sites are exchange-broadened beyond detection [79]. When TFP is added these sites appear as a broad signal at -95 ppm (this is in line with the reduction in the metal ion exchange rate observed by ^{43}Ca NMR). At the same time the "high affinity signal" at -110 ppm broadens and shifts into the other "high affinity signal" at -107 ppm. At $[\text{TFP}]/[\text{TnC}] = 9$ there are two ^{113}Cd signals (which look like two partially superimposed signals) at -98 ppm and -107 ppm corresponding to the former "regulatory" and "high affinity" sites respectively.

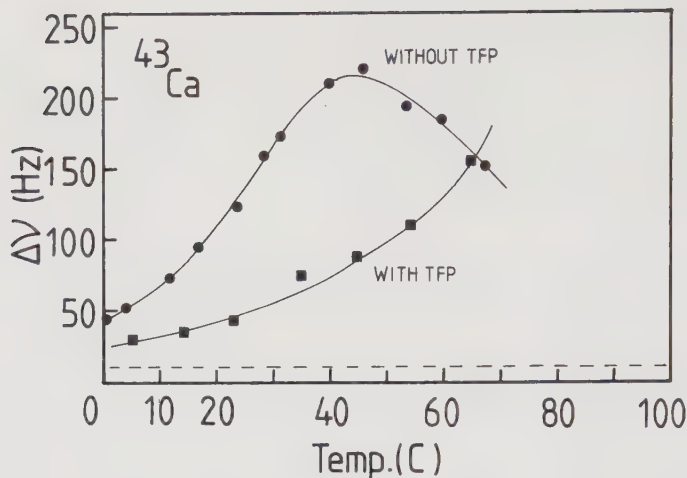


Figure 32. The temperature dependence of the ^{43}Ca NMR signal in the presence and in the absence of TFP. (●) A 5.9 mM CaCl_2 solution containing 0.75 mM TnC. (■) A 3.4 mM CaCl_2 solution in the presence of 0.50 mM TnC and 1.3 mM TFP. The data were recorded at neutral pH.

The results of the experiments quoted above suggest that the binding of TFP to TnC induces a conformational change which in turn affects the exchange rates and geometries both of the "regulatory" sites and at least one of the "high affinity" sites. The binding of TFP to different proteins will be discussed in greater detail in the next chapter.

5.2.5 Metal Ion Binding to TnC

The lower practical concentration limit of ~ 1 mM for ^{43}Ca and ^{25}Mg NMR sets an upper limit of $\sim 10^4 \text{ M}^{-1}$ for the association constants that can be determined. The NMR method is consequently not expected to give good estimates for the Ca^{2+} binding constants to the "high affinity" and "regulatory" sites of TnC. For Mg^{2+} the situation is more favourable, with reported association constants of $3 \pm 1 \cdot 10^3 \text{ M}^{-1}$ for the "high affinity" and "weak" sites [71]. Our value, which also is an average binding constant for these sites, is an order of magnitude smaller ($K_{\text{Mg}} = 5 \pm 1 \cdot 10^2 \text{ M}^{-1}$).

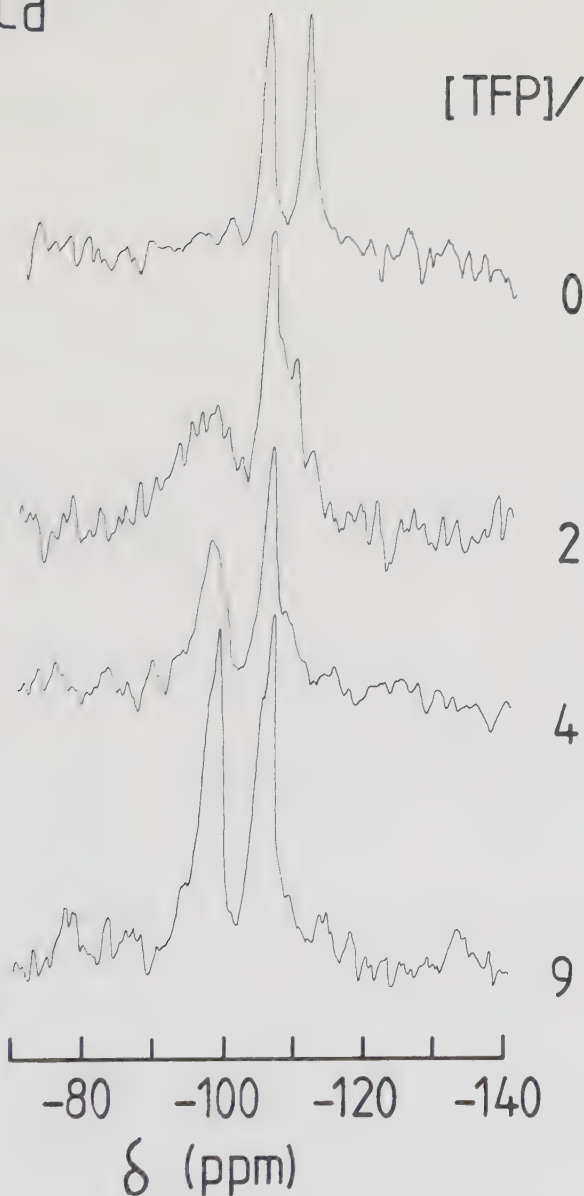
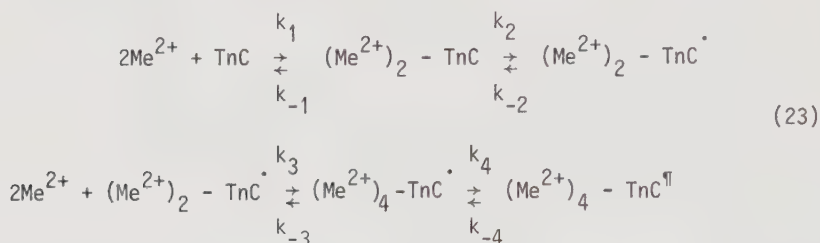
^{113}Cd $[\text{TFP}]/[\text{TnC}]$ 

Figure 33. The ^{113}Cd NMR spectrum of TnC (4 moles of Cd^{2+} /mole TnC) in the presence of different amounts of TFP. (Eva Thulin and Thomas Andersson, results to be published).

The competition experiments described in Figures 27 and 28 show that Ca^{2+} and Mg^{2+} ions bind to the "weak" site(s) with association constants of the order of $5 \cdot 10^2 \text{ M}^{-1}$:

The kinetics of metal ion binding to TnC can, in the absence of Mg^{2+} , be approximated by the following scheme:



The rates of the conformational changes in the protein, steps -2 and -4, have been measured by Johnson et al. using stopped flow fluorimetry [77] and are given in Table 4. The off-rates reported in this thesis are also given for comparison.

Table 4. The rate constants for the protein conformational changes (data within frames) produced by the release of metal ions and the metal ion off-rate (all data at room temperature).

ION	MEDIUM	HIGH AFFINITY SITES	REGULATORY SITES
Ca^{2+}	-	-	$k_{-4} = 300 \text{ s}^{-1}$ $k_{\text{OFF}} = 1000 \pm 100 \text{ s}^{-1}$ $k_{\text{OFF}}(1) \approx 1000 \text{ s}^{-1}$ $k_{\text{OFF}}(2) \approx 50 \text{ s}^{-1}$
Ca^{2+}	excess Mg^{2+}	-	
Mg^{2+}	-	$k_{-2} = 8 \text{ s}^{-1}$? $k_{\text{OFF}} = 8 \pm 5 \cdot 10^3 \text{ s}^{-1}$	-

This comparison reveals that the Ca^{2+} removal from the "regulatory sites" is ca. three times faster than the accompanying conformational change. Assuming that the obtained exchange rate for Mg^{2+} can be taken as a rough estimate of the exchange rate to the "high affinity" sites the difference is much greater, in fact three orders of magnitude. This is in line with the finding that metal binding to the "high affinity" sites elicits a more extensive conformational change than that produced by Ca^{2+} binding to the "regulatory" sites [72-75].

Table 4 also shows that the exchange rate of one of the two "regulatory" sites is reduced by a factor of approximately 20 when Mg^{2+} ions are bound to the "weak" sites. Ca^{2+} binding to these site(s) does not seem to have an effect on the binding properties of the "regulatory" site in question, Figure 23. A simple explanation of this phenomenon is that there is one "weak" site close to one of the "regulatory" sites and that Mg^{2+} binding to the former produces a local conformational change which only affect the proximal "regulatory" site. A similar situation has been found for one of the Ca^{2+} binding sites of carp parvalbumin ($\text{pI} = 4.25$) [80].

5.3. Ca^{2+} BINDING TO BOVINE CALMODULINS

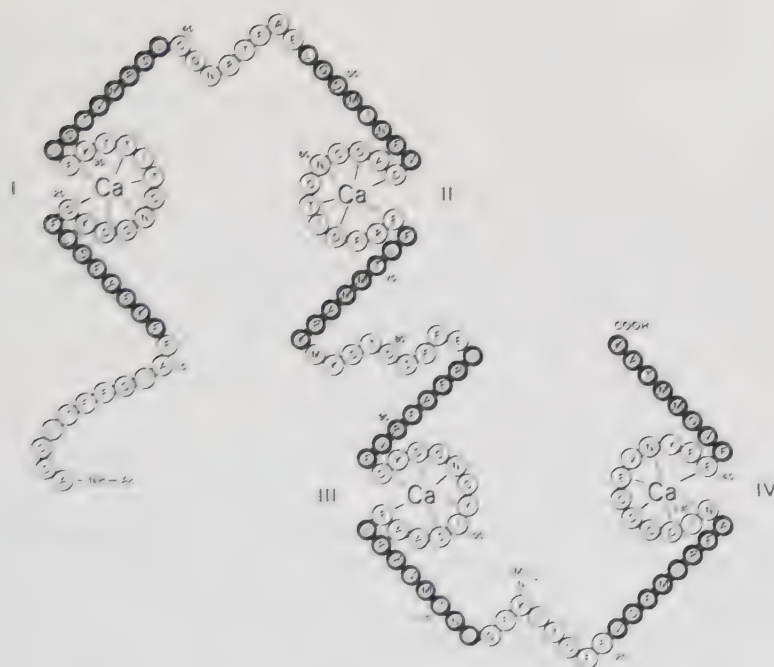
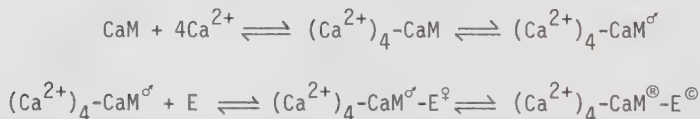


Figure 34. The amino acid sequence of bovine brain calmodulin (A-Ala, 2-Arg, N-Asn, D-Asp, C-Cys, Q-Gln, E-Glu, H-His, I-Ile, L-Leu, K-Lys, M-Met, F-Phe, P-Pro, S-Ser, T-Thr, W-Trp, Y-Tyr, V-Val). The Ca^{2+} binding sites are tentatively assigned from sequence analogies with carp parvalbumin ($\text{pI} = 4.25$) [81]. From ref. 82.

5.3.1. Introduction

Calmodulin is a low molecular weight ($M_r \approx 16700$) thermostable protein. It consists of a single polypeptide chain whose amino acid composition is characterized by a large number of acidic residues, a lack of Cys and Trp and the presence of a residue of the unusual amino acid trimethyllysine. Calmodulin belongs to the same group of Ca^{2+} binding proteins as parvalbumin and troponin C. The crystal structure of calmodulin has not yet been determined. However a comparison of the amino acid sequence of calmodulin with that of parvalbumin suggests the presence of four Ca^{2+} binding sites [83, 84,81].

Like cAMP, calmodulin is a universal regulator that is neither tissue specific nor species specific, that is extremely well conserved during evolution and that affects a large number of cellular events. The regulatory action of calmodulin represents a novel type of enzyme regulation where a monomeric protein is triggered by Ca^{2+} ions to associate with and then modulate the activity of a large number of enzymes. The control process is thought to occur in two stages.



Among the enzymes and processes so far known to be stimulated by the Ca^{2+} -calmodulin complex are [82, 85-88].

1. Cyclic nucleotide phosphodiesterase
2. Brain adenylate cyclase
3. The ATPase of erythrocyte plasma membrane
4. Phosphorylase b kinase
5. NAD^+ kinase in plants
6. Assembly - disassembly of microtubules
7. Protein kinase

The list will certainly expand immensely and perhaps in the future biochemists will ask the question "which enzymes are not under calmodulin control?"

From the list above it can be seen that the Ca^{2+} - calmodulin complex may act in two ways:

1. Directly on an effector system (such as an ATPase)
2. Indirectly on a regulator system (such as the cAMP system)

This situation is shown diagrammatically in Figure 35.

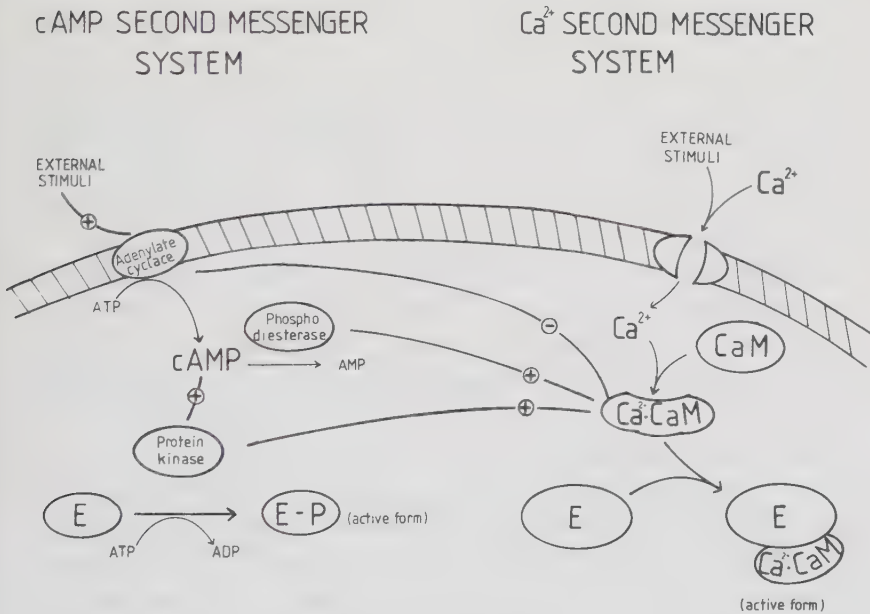


Figure 35. The complex action of calmodulin.

Most studies show that calmodulin stimulates the activity of enzymes but there are also cases of inhibition of enzyme activity caused by calmodulin [86].

An example of indirect regulation that needs special consideration is the modulation of the activities of various enzymes in cAMP systems. This results in a coupling between the two second messenger systems

cAMP and Ca^{2+} . A coupling of this sort appears to be of considerable importance for an understanding of cellular regulation.

The binding of Ca^{2+} to calmodulin has been the subject of several studies (see Table 5). While the relative numbers of high and low affinity sites are somewhat inconsistent most of these studies indicate that calmodulin binds 4 moles of Ca^{2+} /mole protein. ^{113}Cd and ^{43}Ca NMR studies indicate however that there are two sites that bind Ca^{2+} with high affinity (the "high affinity" sites) and two sites that bind Ca^{2+} with lower affinity (the "low affinity" sites) [98, Articles V and VI].

On binding of Ca^{2+} , calmodulin undergoes a large conformational change accompanied by a 5-10 % increase in the α -helical content as determined by circular dichroism (CD) and optical rotary dispersion (ORD) [99, 91-93]. Far UV Cd measurements have shown that the major conformational change occurs at less than full occupancy of the four Ca^{2+} binding sites while other investigations indicate that there may be two (or more) Ca^{2+} dependent transitions.

The first report of competition between Ca^{2+} and other divalent cations was made by Lin et al. [89]. Competition between Ca^{2+} and Mg^{2+} has since then been studied by Woff et al. [91] and Haiech et al. [97] using equilibrium dialysis techniques. The general picture that appears from these studies is that Mg^{2+} competes with Ca^{2+} for all four Ca^{2+} binding sites, however with a much lower (10^2 - 10^3 times) affinity. Seamon [100] reported from ^1H NMR measurements that the spectral changes observed when Mg^{2+} is added to apo-calmodulin are similar, but not identical to those found for Ca^{2+} .

5.3.2. Ca^{2+} Binding to Calmodulin

The measurements described in this chapter were made on calmodulin from two different sources: bovine brain and bovine testis. Bovine brain calmodulin (B-CaM) and bovine testis calmodulin (T-CaM) were prepared as described in ref. [98]. Although these proteins are expected to have very similar if not identical amino acid sequences [81]

small differences in the ^{113}Cd and ^{43}Ca NMR spectra are found [98, this thesis]. At the present stage we cannot rule out that the small differences observed are due to the two different preparation procedures.

Ca^{2+} Titration

The presence of calmodulin (CaM) in a $^{43}\text{Ca}^{2+}$ solution at neutral pH causes significant broadening of the ^{43}Ca NMR signal. Figure 1 in article V shows the ^{43}Ca NMR line width as a function of the $[\text{Ca}^{2+}]/[\text{T-CaM}]$ ratio. As for TnC the line width remains constant (~ 800 Hz) up to a $[\text{Ca}^{2+}]/[\text{T-CaM}]$ ratio of ~ 2 , and the ^{43}Ca signal is shifted ~ 10 ppm downfield relative to "free" $^{43}\text{Ca}^{2+}$. Under the conditions used only the two "high affinity" sites are populated and the observed ^{43}Ca NMR signal derives from $^{43}\text{Ca}^{2+}$ ions bound to these sites. As for the TnC case, measurements of the longitudinal relaxation rate and the transverse relaxation rate (obtained from the line width) yield a value for the correlation time of $\tau_c = 8 \pm 2$ ns at 23°C , Article VI. This value represents the rotational correlation time of the entire protein molecule. Calculations from the correlation time and the transverse relaxation rate give a quadrupole coupling constant of $\chi = 1.15 \pm 0.05$ MHz.

When the $[\text{Ca}^{2+}]/[\text{T-CaM}]$ ratio is increased the ^{43}Ca line width decrease and the observed ^{43}Ca NMR signal is an average exchange-broadened signal due to Ca^{2+} ions bound to mainly one of the two "low affinity" sites (see below). An analysis of the data results in a lower limit of the association constant, $K_{\text{Ca}}^{\text{LAS}}$, for these sites of $\sim 10^4 \text{ M}^{-1}$.

Temperature Dependence

Figure 36 shows the temperature dependence of the ^{43}Ca NMR signal for Ca^{2+} solutions at neutral pH containing: a) testis calmodulin ($[\text{Ca}^{2+}]/[\text{T-CaM}] = 4.8$) and b) brain calmodulin ($[\text{Ca}^{2+}]/[\text{B-CaM}] = 7.5$). At the concentrations used the observed ^{43}Ca signal is broadened due to Ca^{2+} binding to the "low affinity" sites. The temperature dependences shown in Figures 36a and 36b indicate typical transitions from slow exchange

(low temperature) to fast exchange (high temperature). However, a model with two independent sites with the same exchange rates, k_{off} , did not fit the data. A model with two "low affinity" sites having exchange rates differing by a factor of ~ 40 (at room temperature) was found satisfactory for both the brain and testis proteins, see Table 6 (see Figure 4 and section 3.2.2.).

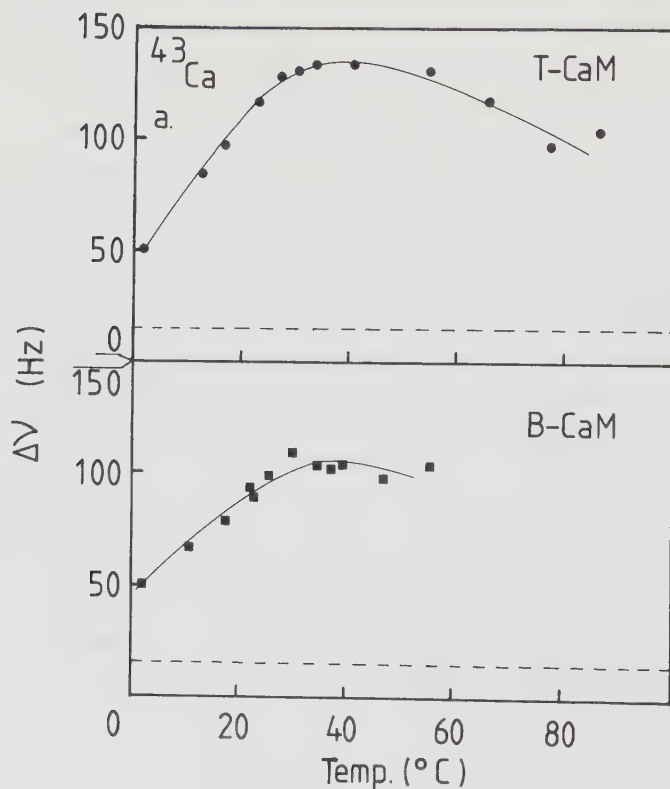


Figure 36. The temperature dependence of the ^{43}Ca NMR line width for a) a 4.3 mM CaCl_2 solution at pH 7.0 containing 0.9 mM T-CaM and for b) a 3.0 mM CaCl_2 solution at pH 7.1 containing 0.4 mM B-CaM. The results of the data analysis are given in Table 6.

pH Dependence

The pH dependence of the ^{43}Ca NMR line width is seen in Figure 37 for CaCl_2 solutions (23°C) containing B-CaM and T-CaM. An analysis of the relaxation data shows that (unlike TnC) the pH dependence can be simulated using a single one-protonation step. The values of the apparent pK values obtained are however different for the brain (4.8) and testis (4.4) proteins. At the Ca^{2+} and protein concentrations used only the "low affinity" sites would be probed. From the exchange rates obtained from the temperature dependence at neutral pH it can be calculated that at 23°C the observed ^{43}Ca broadening will be due to Ca^{2+} ions bound to one of the two "low affinity" sites, namely the one with the fastest exchange rate (see Figure 3). The information obtained from the pH dependence in Figure 37 essentially describes the $\text{H}^+ - \text{Ca}^{2+}$ competition for this site (unless the relative magnitudes of the exchange rates for the two "low affinity" sites are markedly different at lower pH values).

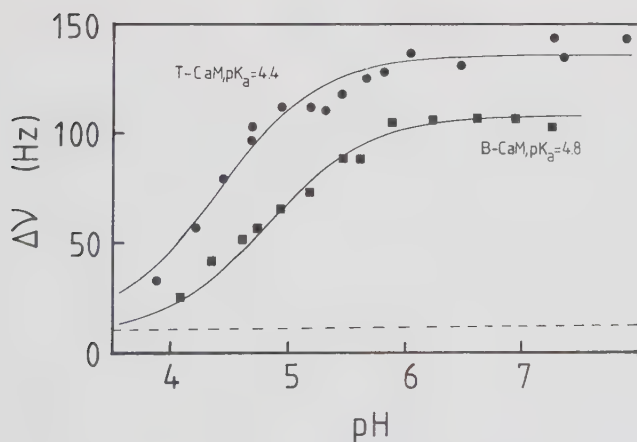


Figure 37. The pH dependence for (●) a 4.3 mM CaCl_2 solution containing 0.9 mM T-CaM and (■) a 3.1 mM CaCl_2 solution containing 0.4 mM B-CaM. The measurements were made at 23°C .

Table 5. A summary of the Ca^{2+} binding parameters obtained from various equilibrium dialysis studies of calmodulin

AUTHORS:	SOURCE	Temp.	CONDITIONS USED		pH	BINDING PARAMETERS	
			Buffer			No. of sites	Binding constants (M^{-1})
Teo & Wang [111]	Bovine heart	24°C	25 mM Tris HCl			1	$3 \cdot 10^5$
			25 mM imidazole			2-3	$8 \cdot 10^4$
Lin et al. [89]	Bovine brain	4°C	3 mM Mg^{2+}		8.0	3	$2.8 \cdot 10^5$
			25 mM Tris			1	$5.6 \cdot 10^4$
Watterson et al. [90]	Bovine brain	25°C	100 mM imidazole	7.0		2	$9.1 \cdot 10^5$
						1-2	$1.2 \cdot 10^3$
Wolff et al. [91]	Bovine brain	25°C	10 mM Tris	7.4		3	$5.0 \cdot 10^6$
						1	$1.0 \cdot 10^6$
Dedman et al. [92]	Rat testis	4°C	100 mM KCl		6.5	4	$4 \cdot 10^5$
			20 mM imidazole				
Klee [93]	Porcine brain	26°C	0.1 mM EGTA		8.0	2	$2.5 \cdot 10^5$
			10 mM Tris			2	$8.3 \cdot 10^4$
Jarret & Kyte [94]	Human erythrocyte	4°C	50 mM NaCl		7.0	4	$1.4 \cdot 10^5$
			10 mM imidazole				(+ additional weaker)
Crouch & Klee [96]	Bovine brain	25°C	100 mM KCl		7.5	4	$1.8 \cdot 10^5$
			10 mM Hepes				(cooperativity)
Haiech et al. [97]	Bovine brain	25°C	150 mM KCl		7.6	4	$10^5 - 10^7$
			100 mM Hepes				(cooperativity)
McCubbin et al. [95]	Bovine brain	20°C	50 mM KCl		7.3	-	$1.3 \cdot 10^7$
			100 mM MOPS			-	$1.1 \cdot 10^4$
			1 mM EGTA				

Table 6. The binding and NMR parameters obtained for CaM and Ca^{2+} .

PROTEIN	SITE	$K(\text{M}^{-1})$	K_{OFF} at 23°C (s^{-1})	pK_a	$\chi(\text{MHz})$
T-CaM	Fast	$>10^4$	1100 ± 300	4.4	1.1 ± 0.1
	Slow	-	20-50	-	
B-CaM	Fast	-	1500 ± 500	4.8	1.0 ± 0.1
	Slow	-	40-100	-	

5.3.3. Phenothiazine Binding to Calmodulin

Calmodulin is known to bind antipsychotic tranquillisers such as phenothiazines, in the presence of Ca^{2+} . A study of Levine and Weiss [101] points out that two moles of phenothiazine per mole CaM are bound with high affinity ($K_a \sim 10^6 \text{ M}^{-1}$). CaM is also known to bind additional drug molecules with much lower affinity [101]. The binding of phenothiazines to CaM has been shown to inhibit the regulatory action of the protein and it has been suggested that this is the mechanism through which this class of drugs exerts its pharmacological action [102]. This view has however recently been opposed by Norman et al. [103] who showed that there was no correlation between the clinical efficacy of drug isomers and their ability to inhibit the activation of phosphodiesterase by CaM. These authors reported that there was a good correlation between the octanol/water partition coefficient of the drug and its inhibitory effect. It was therefore concluded that the phenothiazine derivatives are bound to a hydrophobic site on the CaM molecule. Additional support for this view has been obtained by Laporte et al. [104] and by recent ^{113}Cd measurements in our laboratory (Anita Andersson, unpublished results).

The effect of binding trifluoperazine (one of the most effective phenothiazines) to CaM has been studied by Forsén et al. [98] using ^{113}Cd NMR. From these measurements it was concluded that binding of trifluoperazine (TFP, see Figure 38) causes a conformational change affecting all four of the Ca^{2+} binding sites. The most conspicuous feature in this study was the appearance of the ^{113}Cd signals from the two "low affinity" sites. This demonstrated that TFP binding to CaM also causes a considerable reduction in metal ion exchange rates for these sites. TFP binding to CaM has since then also been studied by ^1H NMR [105].

TFP Addition to Calmodulin

Figure 39 shows the effect of TFP on the ^{43}Ca NMR spectrum at 23°C . Under the experimental conditions used the observed ^{43}Ca broadening is sensitive only to the binding of Ca^{2+} to one of the "low affinity" sites, the one with the fastest Ca^{2+} exchange. The data in Figure 39

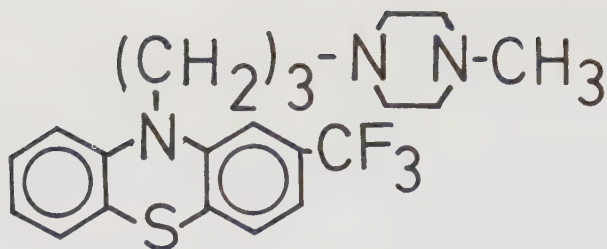


Figure 38. Trifluoperazine (TFP).

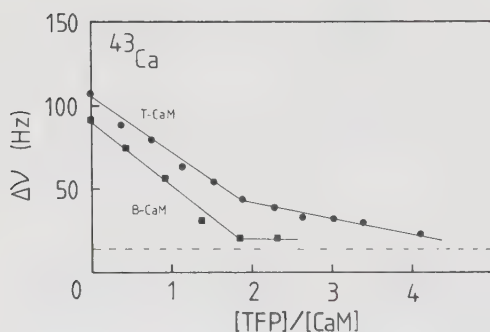


Figure 39. The ^{43}Ca NMR line width as a function of the $[\text{TFP}]/[\text{CaM}]$ ratio for (■) a 3.0 mM CaCl_2 solution, pH 7.0 containing 0.40 mM B-CaM (●) a 3.0 mM CaCl_2 solution, pH 7.0 containing 0.44 mM T-CaM. The data was recorded at 23°C .

shows that addition of TFP decreases the ^{43}Ca broadening up to a $[\text{TFP}]/[\text{CaM}]$ ratio of ~ 2 . This decrease is due to a reduction in the Ca^{2+} exchange rate (~ 100 fold) to the site observed. This is evident from the temperature dependence of the ^{43}Ca NMR signal in the presence of TFP and CaM, Figure 40. This is in agreement with the conclusions drawn from ^{113}Cd measurements [98], namely that the binding of TFP

causes an extensive conformational change which decreases the off-rate for the metal ions, possibly due to an increase in the metal ion affinity.

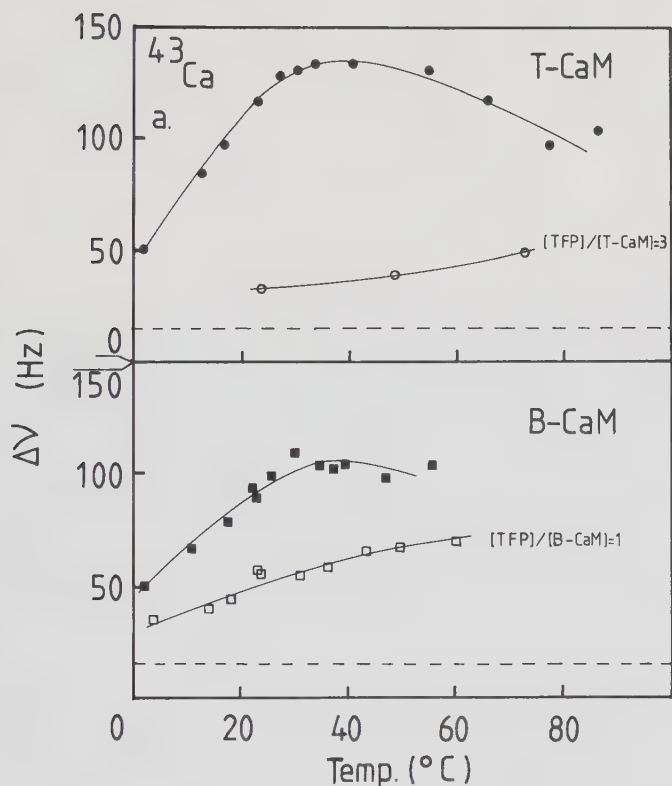


Figure 40. The temperature dependence of the ^{43}Ca NMR line width for: a) ($\bullet$) a 4.3 mM CaCl_2 solution at pH 7.0 containing 0.9 mM T-CaM, ($\circ$) a 3.0 mM CaCl_2 solution at pH 7.0 containing 0.4 mM T-CaM and 1.3 mM TFP. b) ($\blacksquare$) a 3.0 mM CaCl_2 solution at pH 7.1 in the presence of 0.4 mM B-CaM, ($\square$) 3.0 mM CaCl_2 solution, pH 7.0. containing 0.4 mM B-CaM and 0.4 mM TFP.

The data in Figure 39 show that both bovine brain and testis proteins bind two TFP molecules with high affinity. This observation is in agreement with equilibrium dialysis data [78], ^1H NMR [105] and ^{113}Cd measurements [98]. The testis protein however seems to bind additional TFP molecules, which affects the remaining ^{43}Ca NMR line width. In this respect the brain and testis proteins show a significant difference.

The binding of these additional TFP molecules has been observed by equilibrium dialysis [78] and ^{113}Cd NMR (Eva Thulin, unpublished results). The ^{113}Cd NMR measurements show similar but not identical spectral changes for the bovine and testis proteins on adding TFP.

Conclusions

In most other ^{43}Ca NMR experiments (except the measurements of the pH dependence, Figure 37) the bovine testis and brain calmodulins behave similarly. The TFP binding studies and the ^{43}Ca pH dependence suggest however that there are some significant differences. At the time of writing we have no well-founded interpretation of this. One explanation is that the two proteins have slightly different amino acid sequences. Another interpretation which cannot be ruled out is that one of the proteins (most likely B-CaM) is contaminated by a small molecule or in some other way modified during the preparation procedure.

The addition of TFP to other Ca^{2+} binding proteins such as carp parvalbumin $\text{pI}=4.25$ (M. Sward, ^{113}Cd NMR, unpublished results), calf brain S-100 and porcine pancreatic phospholipase A_2 (^{43}Ca NMR, unpublished results) does not affect the NMR parameters. So far only troponin C and calmodulin have shown strong binding of TFP. The binding seems however not to be limited to phenothiazine derivatives. For example other hydrophobic drug types and fluorescent dyes bind to CaM although with lower affinities [103, 104; A. Andersson, unpublished results]. It is important to note that both TnC and CaM have to be in their fully Ca^{2+} -loaded conformation in order to account for the high affinities drug binding [78].

The current picture is that Ca^{2+} binding to TnC and CaM causes an extensive conformational change which results in the exposure of a domain of considerable hydrophobic character. This hydrophobic area is considered to participate in the protein-protein interaction of TnC and CaM in their respective protein complexes. The non-polar ligands discussed above may also bind to these domains. This would provide a simple interpretation of the inhibition of the regulatory action of calmodulin caused by these ligands: when these drug derivatives are bound to the CaM-enzyme interaction area and the association between the two molecules are hindered and thus the regulatory action is inhibited.

5.3.4. Summary of Ca^{2+} Binding to CaM

The lower practical concentration limit of ~ 1 mM in this type of study prevent us from estimating Ca^{2+} binding constants for the four cation sites of CaM. Ca^{2+} binding to calmodulins has been the subject of several investigations (Table 5). Although all of these studies show 4 strong Ca^{2+} sites there are significant differences in the affinities of the four sites. There does not seem to be any correlation between the experimental conditions used and the resulting binding parameters. Most of these studies were performed using equilibrium dialysis techniques and the data was treated by linearised plots of Scatchard type. This form of data analysis has often been criticised for being inaccurate [106].

^{113}Cd NMR and ^{43}Ca NMR data [98, this thesis] indicate the presence of two non-identical "high affinity" sites analogous to those found in TnC and two "low affinity sites" having exchange rates differing by a factor of ~ 30 . A lower limit of $1 \cdot 10^4 \text{ M}^{-1}$ for the association constant of the "low affinity" site observed by ^{43}Ca NMR is given by our study. The binding constant to the other site is approximately 30 times greater, assuming that the Ca^{2+} on-rates are the same for the two sites.

The Ca^{2+} binding properties of calmodulin very much resemble those for troponin C in the presence of excess amounts of Mg^{2+} .

4. CATION BINDING TO PANULIRUS INTERRUPTUS HEMOCYANIN

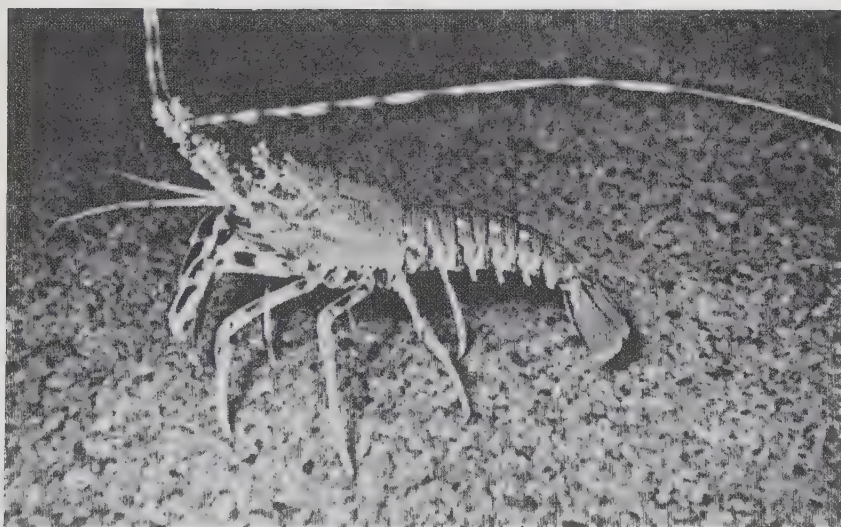


Figure 41. The spiny lobster Panulirus.

5.4.1. Introduction

The hemocyanins serve as oxygen carriers in arthropods and molluscs. Unlike the hemoglobins, that contain iron, the hemocyanins contain copper which accounts for their characteristic blue colour in the oxygenated state. One molecule of oxygen is bound per two copper atoms. Hemoglobins are present in blood cells but the hemocyanins are found in solution in the hemolymph [107].

The hemocyanins found in molluscs are quite different from those of arthropods. Molluscan hemocyanins consist of polypeptide chains ($M_r = 2-3 \cdot 10^5$) carrying 7-8 oxygen binding sites each. Arthropod hemocyanins are made up of smaller subunits ($M_r = 7-8 \cdot 10^4$) each carrying one oxygen binding site [108]. Many hemocyanins occur as glycoproteins with 1-10 % of carbohydrate attached to the molecule.

The hemocyanins form a remarkable variety of aggregates. The state of aggregation depends on the experimental conditions used. The general conditions which promote aggregation are similar for both arthropod and molluscan hemocyanins: pH values around neutrality and the presence of cations such as Ca^{2+} and Mg^{2+} and Na^+ [108]. In addition the state of aggregation can be affected by oxygen binding [108].

Oxygen binding to hemocyanins has in many cases been shown to be co-operative and to be affected by the pH and the presence of ions such as Na^+ , Ca^{2+} and Mg^{2+} [108].

The results presented above show that cation binding to hemocyanins may be important for their functional properties. In this project we have chosen to study the relatively small hemocyanin from the spiny lobster Panulirus interruptus (Figure 41). This protein is made up of 6 monomers ($M_r \approx 7.5 \cdot 10^4$) each containing one oxygen binding site. The subunits are arranged into two triangles which lie in parallel planes and are rotated approximately 25° around the common threefold symmetry axis [109] (Figure 42). The native hexamer is stable between pH ~ 6.2 and ~ 8.5 . In the presence of millimolar Ca^{2+} concentrations (or Na^+ concentrations of the order of 0.1 - 1 M) the range of stability is extended by 1 - 2 pH units on the alkaline side.

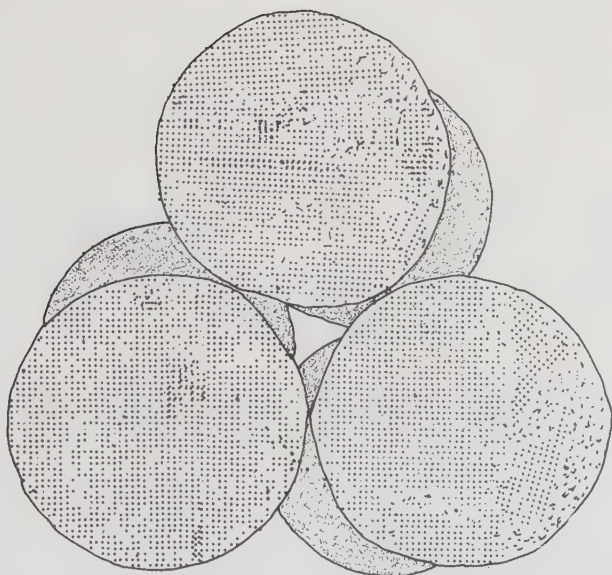


Figure 42. The arrangement of the subunits in the native hexameric form of Panulirus interruptus hemocyanin [109].

5.4.2. Summary

Cation binding to Panulirus interruptus hemocyanin is described in Article VII. Binding sites on the hemocyanin molecule were probed using ^{43}Ca NMR and ^{23}Na NMR. ^{43}Ca NMR reveals the presence of several (10 ± 7) sites per subunit. These sites bind Ca^{2+} and Mg^{2+} with approximately equal affinities, $K_{\text{Ca}}^W \approx K_{\text{Mg}}^W = 1 \cdot 10^3 \text{ M}^{-1}$. ^{23}Na on the other hand probes a few (1-2) sites per subunit. These sites bind Ca^{2+} more strongly than Mg^{2+} ($3 \pm 1 \cdot 10^4 \text{ M}^{-1}$ compared to $10 \pm 2 \cdot 10^3 \text{ M}^{-1}$).

The values of the correlation times obtained for ^{23}Na and ^{43}Ca indicate considerable mobility at the cation binding site.

The general features of the cation binding suggest that cations are bound to carboxylate groups on the surface of the protein.

6. CONCLUSIONS

The main goal of this thesis has been to characterise the ion binding properties of proteins using quadrupole relaxation.

The ion binding to cytochrome c has been studied using ^{35}Cl NMR. Both oxidation states were shown to bind anions such as Cl^- , ClO_4^- , phosphate and iron hexacyanides. This observation is at variance with earlier studies. The ion binding properties of oxidised and reduced cytochrome c were shown to be somewhat different.

NMR is an inherently insensitive spectroscopic technique and much effort has been made to extend the concentration range accessible for this type of study. ^{43}Ca has been considered to be one of the "worst" nuclei from a sensitivity point of view. The use of isotopically enriched chemicals in combination with recent instrumental developments have made ^{43}Ca measurements possible even at millimolar concentrations.

Using ^{43}Ca NMR exchange rates and binding constants have been determined for several Ca^{2+} binding proteins. The method thus shows good promise for the study of this type of problems.

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ARTICLE I

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Ion Binding to Cytochrome *c* Studied by Nuclear Magnetic Quadrupole Relaxation



Ion Binding to Cytochrome *c* Studied by Nuclear Magnetic Quadrupole Relaxation[†]

Thomas Andersson,* Eva Thulin, and Sture Forsén*

ABSTRACT: The enhancement of the $^{35}\text{Cl}^-$ transverse relaxation rate on binding of chloride ions to oxidized and reduced cytochrome *c* has been studied under conditions of variable sodium chloride concentration, temperature, pH, sodium phosphate, iron hexacyanide, and sodium cyanide concentration. The results revealed the presence of a strong binding site(s) for chloride in both oxidized and reduced cyt *c*, with a higher affinity in ferrocytochrome *c*. Competition experiments suggest that these sites also bind iron hexacyanide and

phosphate. Cyanide binding to the iron in ferricytochrome *c* at alkaline and neutral pH was shown to decrease the binding of chloride. The pH dependence of the $^{35}\text{Cl}^-$ relaxation rate has been fitted by using literature pK values for ionizable groups. No indications of Na^+ binding to oxidized and reduced cytochrome *c* have been observed by using $^{23}\text{Na}^+$ NMR. Our results suggest that chloride is bound near the exposed heme edge and that the surface structure or dynamics in this region are different in the two oxidation states.

Eucaryotic cytochrome *c* is a small protein, composed of 103–113 amino acid residues, having an iron porphyrin co-

valently attached to the protein. The biological role of cytochrome *c* is to receive electrons from cytochrome *c*₁ and in turn deliver them to cytochrome oxidase (Dickerson & Timkovich, 1975). The crystal structure of tuna cytochrome *c* has been determined for both oxidation states (Swanson et al., 1977; Takano et al., 1977; Mandel et al., 1977), and no

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significant differences have been observed. However, there is an extensive body of physical and chemical data indicating that the physical properties of cytochrome *c* differ in its oxidized and reduced states (Salemme, 1977). The origin of these differences may possibly be sought in the surface region of the protein molecule, in particular the arrangement of the amino acid side chains. At the present time, several studies indicate that the positively charged residues play an important role in the interaction between cytochrome *c* and the complexes in the respiratory chain (Salemme, 1977; Ng et al., 1977; Smith et al., 1977; Ferguson-Miller et al., 1978).

Any changes involving the surface charges upon reduction should be reflected in differences in the ion binding properties of the two forms. Ion binding to cytochrome *c* has been a widely investigated phenomenon. From electrophoretic studies, Margoliash and co-workers (Barlow & Margoliash, 1966; Margoliash et al., 1970) found that oxidized cytochrome *c* binds chloride and phosphate, while the reduced form only binds phosphate. Sodium was found only to bind to the reduced protein. Margalit and Schejter (Schejter and Margalit, 1970; Margalit et al., 1970; Margalit & Schejter, 1973a,b, 1974) have studied ion binding by redox potential measurements and gel filtration techniques. They came to the conclusion that phosphate and chloride ions bind exclusively to the oxidized protein with a binding constant of about 10^4 M^{-1} . No evidence of sodium binding to the protein was found. Chloride and phosphate binding to reduced cytochrome *c* has, however, been observed by Stellwagen & Shulman (1973a) by using proton NMR. These authors were able to estimate the binding constant ($K_b = 400 \text{ M}^{-1}$) for phosphate to a site in the vicinity of His-26.

The results above are partially contradictory and suggest that ion binding to cytochrome *c* is worthy of further investigation. Nuclear magnetic quadrupolar relaxation spectroscopy is a sensitive and direct method for studying ion binding to macromolecules (Lindman & Forsén, 1976; Forsén & Lindman, 1978), and the work presented here is a $^{23}\text{Na}^+$ and a $^{35}\text{Cl}^-$ NMR investigation of binding of these ions to both oxidation states of horse heart cytochrome *c*.

Experimental Procedure

Materials. Horse heart cytochrome *c* was prepared by the method of Margoliash & Walasek (1967) and further purified on an ion-exchange resin to separate deamidated forms from the native protein. The absence of artifactual forms was checked by gel electrophoresis and carbon monoxide combination (Tsou, 1951). After elution from the ion-exchange column, the protein was dialyzed against triple distilled water for several days in the cold room, and finally deionized on a mixed bed ion-exchange resin. The cytochrome oxidase activity was assayed spectrophotometrically by using the method of Smith & Conrad (1956). By these procedures, we were able to obtain a pure and highly active protein.

Protein concentrations were determined on the basis of the extinction coefficient ϵ $29.5 \text{ cm}^{-1} \text{ mM}^{-1}$ at 550 nm for the reduced protein. A stock solution of the oxidized protein was kept at -20°C and used within 1 month of preparation. Reduced cytochrome *c* was prepared by ascorbate reduction and the salt was removed by dialysis followed by deionization on a mixed bed resin.

Ferricytochrome *c* was carboxymethylated at room temperature by using a buffered solution, pH 7, containing NaCN and bromoacetate (Stellwagen, 1968). An amino acid analysis showed that cytochrome *c* had been modified at Met-65, Met-80, and His-33. The product also showed the characteristic absorbance spectrum of cytochrome *c* with both

methionyl residues alkylated. Reduced carboxymethylated cytochrome *c* was prepared by adding an excess of sodium dithionite.

In the NMR experiments, no buffers were used; pH was adjusted by addition of dilute HCl or NaOH. In the $^{23}\text{Na}^+$ measurements D_2O was used instead of H_2O . All chemicals were of the finest grade available.

Relaxation Measurements. The nuclear magnetic resonance spectra of $^{35}\text{Cl}^-$ and $^{23}\text{Na}^+$ were obtained by using a modified Varian XL-100-15 spectrometer operating in the Fourier transform mode. For the $^{35}\text{Cl}^-$ measurements we used an external proton lock, and for the $^{23}\text{Na}^+$ recordings the magnetic field was internally locked, on the deuterium signal from the solvent. A few measurements were performed on a home-built Fourier transform spectrometer equipped with an Oxford Instruments 6-T wide-bore magnet. The probe temperature was held constant by a stream of dry thermostated nitrogen gas. The spectra were recorded at 28°C unless otherwise stated. The linewidths, $\Delta\nu$, taken at half-height of the absorption signal, are related to the transverse relaxation rate, R_2 , through $R_2 = \pi\Delta\nu$. The error in the measurements was estimated to be 3%.

Theory

For any nucleus with a spin quantum number (I) greater than $1/2$, such as ^{35}Cl and ^{23}Na , the distribution of charge over the nucleus deviates from spherical symmetry, a deviation which can be described in terms of a nuclear electric quadrupole moment (eQ). If the electron distribution about the nucleus has less than cubic symmetry, then an electric field gradient at the nucleus will result which can couple to the nuclear quadrupole moment. Fluctuations in this field gradient coupled to the quadrupole moment can provide an efficient mechanism for nuclear magnetic relaxation, so efficient that, for most quadrupolar nuclei, it is the only relaxation mechanism that need be considered.

For a "free" solvated quadrupolar ion in aqueous solution, quadrupolar relaxation is relatively inefficient. For a quadrupolar ion bound in an asymmetric environment, for example, to a macromolecular binding site, the quadrupole relaxation is usually very efficient—a direct study of the bound ion is then extremely difficult. Studies of the binding of quadrupolar ions to macromolecules are, however, possible if there is a fast (or intermediate) chemical exchange between the bound and "free" ions (Forsén & Lindman, 1978).

If the chemical exchange between the protein sites and the bulk solution is much faster than the transverse relaxation rates at the protein sites, the excess transverse relaxation rate is given by

$$R_{2,e} = R_{2,o} - R_{2,f} = \sum_i p_i R_{2,i} \quad (1)$$

where $R_{2,o}$ is the observed relaxation rate and $R_{2,f}$ is the relaxation rate in the absence of protein, and p_i and $R_{2,i}$ are the fraction of ions bound to and the relaxation rate at site i , respectively. For the case with no competing ions in the solution, the fraction of bound ions is given by

$$p_i = \frac{n_i c_{\text{pr}} K_{i,X}}{1 + K_{i,X}(X)} \quad (2)$$

where n_i is the number of ions bound to site i with the binding constant $K_{i,X}$. The total protein concentration is denoted c_{pr} and (X) refers to the concentration of unbound ions. In the general situation where other ions, L , compete with X for the same binding sites, the average fraction of the ion X bound

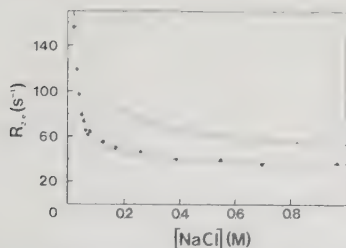


FIGURE 1: The $^{35}\text{Cl}^-$ excess transverse relaxation rate as a function of NaCl concentration in the presence of oxidized (O) and reduced (●) cytochrome *c* at pH 7.3 and 7.2, respectively. The data are normalized to a protein concentration of 3.0 mM. Lines are theoretical curves calculated by using the parameters given in Table I.

to *i* on the protein may be expressed as (Steinhardt & Reynolds, 1969)

$$p_i = \frac{n_i c_{\text{pr}} K_{i,x}}{1 + K_{i,x} (X) + \sum_L K_{i,L} (L)} \quad (3)$$

$K_{i,L}$ is the association constant for the competing ligand *L*, and (*L*) denotes the concentration of the free ions. For a moderately large protein, such as cytochrome *c*, and, for the nuclei and magnetic fields employed in this work, extreme narrowing conditions ($\omega\tau_c \ll 1$) apply (Lindman & Forsén, 1976) and the intrinsic transverse relaxation rate $R_{2,i}$ for a quadrupolar nucleus at site *i* is given by

$$R_{2,i} = \frac{2\pi^2}{5} \chi_i^2 \tau_{cl} \quad (4)$$

where χ_i is the nuclear quadrupolar coupling constant and τ_{cl} is the correlation time describing the reorientation of the electric field gradient at site *i*. τ_{cl} can, in principle, contain contributions from the overall protein rotational motion, chemical exchange, and the internal motion of the bound ion. Equation 1 shows that in the fast exchange limit there are two main parameters determining the relaxation rate, the fraction of bound ions and the intrinsic relaxation rate. The latter is determined by the quadrupole coupling constant and correlation time according to eq 4.

Results and Discussion

Dependence of the $^{35}\text{Cl}^-$ Relaxation Rate on NaCl Concentration. The dependence of the $^{35}\text{Cl}^-$ transverse relaxation rate on chloride concentration at pH near neutrality for the oxidized and reduced forms of cytochrome *c* is shown in Figure 1. The recordings were obtained by using protein concentrations between 1 and 4 mM, and the relaxation rates are corrected to 3.0 mM cytochrome *c* concentration. The data can be interpreted in terms of a two-site model, having one class of high affinity sites (index *i*) and one class of low affinity sites which gives a constant contribution ($\sum_n P_n R_{2,n}$) to the observed relaxation rate. By combining eq 1 and 2, and by making the approximation that the concentration of free chloride ions equals the total concentration, C_{Cl} , of these ions, the excess transverse relaxation rate may be expressed as

$$R_{2,e} = \frac{n_i c_{\text{pr}} K_{i,x} R_{2,i}}{1 + K_{i,x} C_{\text{Cl}}} + \sum_n P_n R_{2,n} \quad (5)$$

The experimental relaxation rates were fitted to eq 5 by using a least-squares fitting program. The results of this operation are given in Table I. Since the number of sites contained in each class is not known, the intrinsic relaxation rate is given as the product $n_i R_{2,i}$. Due to experimental limitations, it is

Table I: Results of the Fitting of the Binding Parameters of Chloride to Oxidized and Reduced Cytochrome *c*

	high-affinity class		low affinity class
	$K_{\text{Cl}} (M^{-1})$	$n_i R_{2,i} (s^{-1})$	$\sum_n P_n R_{2,n} (s^{-1})$
Fe $^{3+}$ -cyt <i>c</i>	17	2.7×10^3	44
Fe $^{2+}$ -cyt <i>c</i>	$\geq 10^2$	9×10^2	33

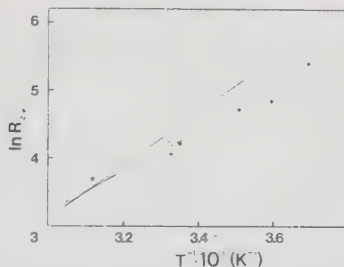


FIGURE 2: The $^{35}\text{Cl}^-$ excess transverse relaxation rate of a 0.10 M NaCl solution as a function of the inverse absolute temperature in the presence of oxidized cytochrome *c* at pH 7.3 (O) and pH 9.2 (□), and at pH 7.2 for reduced cytochrome *c* (●). The relaxation rates are normalized to 3.0 mM protein concentration. The lines are drawn by using a least-squares fitting program.

not possible to determine large binding constants, but it can clearly be stated that chloride binds more tightly to the high affinity sites of reduced cytochrome *c*. The derived intrinsic relaxation rates are much smaller than expected on the basis of $^{35}\text{Cl}^-$ binding studies to other proteins (Lindman & Forsén, 1976). A possible explanation for this is that rapid local motions at the binding sites lead to a reduced effective quadrupolar coupling constant.

The origin of the differences in the observed $^{35}\text{Cl}^-$ excess relaxation rates between the oxidized and reduced forms of cytochrome *c* is at the present time not clear. Since the chloride chemical exchange rate is fast in comparison with the chloride relaxation rate at the protein binding site (cf. following section), this rules out differences in chemical exchange rates. Upon oxidation, the heme charge increases by one unit but simple calculations show that this will not have any significant effect on the quadrupole relaxation of chloride ions bound near the heme crevice. Neither is any significant paramagnetic contribution to the $^{35}\text{Cl}^-$ transverse relaxation rate expected on the basis of substituting reasonable parameters into the Solomon-Bloembergen equations (Dwek, 1973).

Dependence of the $^{35}\text{Cl}^-$ Relaxation Rate on Temperature. If the exchange of chloride ions is rapid enough to influence the $^{35}\text{Cl}^-$ signal from the bulk solution, the excess relaxation rate can be interpreted in terms of an average of the intrinsic relaxation rates of the different sites, weighted according to their population. It can be shown (Lindman & Forsén, 1976) that, in the fast exchange limit, a plot of $\ln R_{2,e}$ vs. T^{-1} is linear with a positive slope. Figure 2 shows the results obtained for 0.10 M NaCl solutions at pH 7.3 and 9.2 for oxidized cytochrome *c*, and at pH 7.2 for reduced cytochrome *c*. Protein concentrations between 1 and 2.5 mM were used, but the data are normalized to 3.0 mM. The results indicate chloride ion exchange to be fast, confirming the validity of eq 1.

Dependence of the $^{35}\text{Cl}^-$ Relaxation Rate on pH. The effect of pH on the $^{35}\text{Cl}^-$ relaxation rate for both oxidation states of cytochrome *c* is shown in Figure 3. The results were

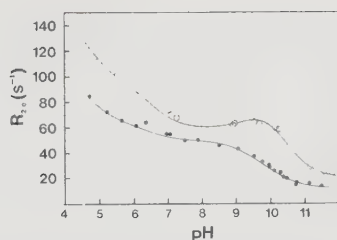


FIGURE 3: The pH dependence of the $^{35}\text{Cl}^-$ excess transverse relaxation rate for a 0.30 M NaCl solution containing oxidized (O) and reduced (●) cytochrome *c*. The data are normalized to a protein concentration of 3.0 mM. The solid lines are theoretical curves drawn by using the parameters appearing in Table II.

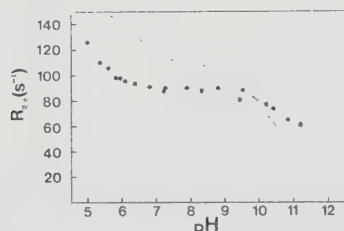


FIGURE 4: The pH dependence of the $^{35}\text{Cl}^-$ excess transverse relaxation rate for a 0.3 M NaCl solution containing oxidized (O) and reduced (●) carboxymethylated cytochrome *c*. The data are corrected to 3.0 mM protein concentration. Lines are theoretical curves drawn by using the parameters given in Table II.

obtained in 0.3 M NaCl solutions containing 2.2 mM ferri-cytochrome *c* and 3.4 mM ferrocytochrome *c*, respectively. The pH was adjusted by adding small amounts of dilute NaOH and HCl. The data are normalized to a protein concentration of 3.0 mM.

Again we can clearly observe a difference between the oxidized and reduced forms. The most conspicuous feature in Figure 3 is the "hump" in the $^{35}\text{Cl}^-$ excess relaxation rate for the oxidized form at a pH around 9.5. It is known from spectroscopic studies that oxidized cytochrome *c* exists in five distinct conformational states. The physiologically active state, usually denoted state III, is stable between pH 2.5 and pH 9.5 where it undergoes a transition to state IV (Dickerson & Timkovich, 1975). The disappearance of the methionine methyl signal in the proton NMR spectrum during this alkaline isomerization indicates that methionine-80 is displaced as the sixth heme ligand (Redfield & Gupta, 1971). The heme iron remains, however, in a low spin state, indicating that another strong field ligand, possibly Lys-79 but cf. Pettigrew et al. (1976), coordinates to the heme iron. If cytochrome *c* is carboxymethylated at Met-80, a lysine, probably the same as in state IV, is already coordinated to the heme iron at neutral pH. Figure 4 shows the pH dependence of oxidized and reduced carboxymethylated cytochrome *c*. It is seen that the oxidized protein does not show any increase in the $^{35}\text{Cl}^-$ relaxation rate in the pH range 9–10. The "hump" in Figure 3 must obviously be related to the conformational transition between states III and IV, resulting in either an increased binding to Fe^{3+} -cyt *c*, an increased correlation time, and/or an increased quadrupole coupling constant of a binding site. The temperature dependence of Fe^{3+} -cyt *c* at pH 9.2 rules out

Table II: Results of the Fitting of the pH Dependence of the Relaxation Rates for Native and Carboxymethylated Cytochrome *c*

	$\text{pK}_{a,i}$	$R_{2,i}$ (s^{-1})	assignment
Fe^{3+} -cyt <i>c</i>	10.4	70	Lys residue(s)
	9.5	-31	alkaline isomerization
	6.5	38	His-33
	4.6	60	carboxylate residues
Fe^{2+} -cyt <i>c</i>	10.2	18	Lys residue(s)
	9.4	20	Lys-79?
	6.4	14	His-33
	4.6	45	carboxylate residues
Fe^{3+} CM-cyt <i>c</i>	10.2	65	Lys residue(s)
	6.4	45	?
Fe^{2+} CM-cyt <i>c</i>	10.4	35	Lys residue(s)
	4.4	220	carboxylate residues

the possibility of chemical exchange effects. It is not straightforward to separate these different factors. A large number of positive surface charges around the heme edge are due to lysyl groups that have considerable flexibility. A theoretical study of the relaxation of quadrupolar ions attached to flexible binding sites (Bull, 1978) has shown that the observed apparent correlation time and quadrupole coupling constant depend in a complex way on the mode of internal motion. Therefore, it can not be ruled out that the alkaline isomerization is accompanied by a conformational change in the backbone that influences the freedom of motion of these positive side chains in such a way as to cause increased relaxation rates of interacting halide ions. On the other hand, the alkaline isomerization may be accompanied by a change in the protein fold in such a way that a new anion binding site(s) is created or the affinity of a weak binding site is considerably increased. The recently observed effect of different ions on the transition III $\rightarrow$ IV provides evidence favoring the latter explanation. In the presence of increasing concentration of ClO_4^- , an ion that interacts strongly with cytochrome *c*, the pK value is shifted toward lower pH values, as would be expected if state IV has a higher affinity for ClO_4^- than state III (J. Ångström, K.-E., Falk, and T. Andersson, unpublished results).

The binding of ions to protein molecules is primarily due to electrostatic interactions between the ion and charged residues on the protein. It is sometimes possible to fit the pH dependence of the relaxation rate by using known pK values of the ionizable groups on the macromolecule (Halle & Lindman, 1978). Shaw & Hartzell (1976) have determined these pK values for ferri-cytochrome *c* using hydrogen ion titrations, and they are in good agreement with pK values determined by other techniques. For reduced cytochrome *c*, to the authors' knowledge, no similar investigation of the ionizable groups has been carried out. Proton NMR shows that histidine-33 has nearly the same pK value in the two oxidation states (Cohen et al., 1974; Cohen & Hayes, 1974). The experimental relaxation rates were fitted to the equation

$$R_{2,e} = \sum_i \frac{R_{2,i}}{1 + 10^{(\text{pH} - \text{pK}_{a,i})}} \quad (6)$$

where $R_{2,i}$ is the contribution to the relaxation rate from chloride ions exchanging with site *i*. The pK value of this site, denoted $\text{pK}_{a,i}$, was taken from the literature. For reduced cytochrome *c*, we needed to make only very small corrections to these pK values in order to obtain a good fit. The "hump" in the relaxation rate was introduced by using a negative $R_{2,i}$ corresponding to the pK of the alkaline isomerization. The

¹ Abbreviations used: Fe^{3+} -cyt *c*, ferri-cytochrome *c*; Fe^{2+} -cyt *c*, ferrocytochrome *c*.

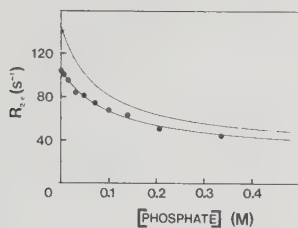


FIGURE 5: The $^{35}\text{Cl}^-$ excess transverse relaxation rate of a 0.073 M NaCl solution, pH 7.0, containing 3.1 mM oxidized (O) and reduced (●) cytochrome *c*, as a function of the total concentration of added phosphate. Lines are theoretical curves drawn by using the calculated relative binding constants: $K'/K = 0.8$ for oxidized and $K'/K = 1.7$ for reduced cytochrome *c*.

results of the fitting procedures are shown as the solid lines in Figures 3 and 4 with the corresponding parameters appearing in Table II. The data indicate that His-33 is involved in chloride binding in both oxidation states. It is also seen that the decrease in the relaxation rate above pH 8 for the carboxymethylated cytochrome can be fitted by using only one pK value, whereas the native reduced cytochrome *c* requires at least two pK values. This can be explained by the fact that a lysyl residue (probably Lys-79) in the carboxymethylated protein is coordinated to the heme iron and is no longer able to participate in the binding of chloride.

Competition between Phosphate and Chloride. Several studies have pointed out that phosphate binds to cytochrome *c*. Margolish and co-workers (1970) reported that phosphate binds to both oxidation states at neutral pH. On the other hand, Margalit & Schejter (1973b) state that phosphate binds exclusively to the oxidized form of cytochrome *c*.

Figure 5 shows the excess $^{35}\text{Cl}^-$ relaxation rate of 0.073 M NaCl solutions, pH 7.0, containing oxidized and reduced cytochrome *c*, as a function of the total concentration of added phosphate. The measurements in Figure 5 were performed on a 6-T spectrometer. The sample temperature was somewhat lower than for the other experiments (23 °C compared with 28 °C), resulting in a small relative signal broadening. Our results are most easily interpreted in terms of phosphate and chloride competition for common sites in both oxidized and reduced cytochrome *c*. The experimental relaxation rates were fitted to the equation

$$R_{2,e} = \frac{n_i C_{pr} K_i R_{2,b}}{1 + K'_i/(\text{PO}_4) + K_i/(\text{Cl}^-)} + \sum_{n \neq i} P_n R_{2,n} \quad (7)$$

where n_i is the number of ions bound at site *i*, K_i and K'_i are the intrinsic association constants for chloride (given in Table I) and phosphate, respectively, $R_{2,b}$ is the relaxation rate for the bound chloride ion, and C_{pr} the total protein concentration. The concentrations of free phosphate and chloride ions are denoted (PO_4) and (Cl^-) and they can, under the experimental conditions used, be approximated by the total concentrations of these ions. The second term in eq 7 describes the contribution to the observed $^{35}\text{Cl}^-$ relaxation rate from sites which are not affected upon addition of phosphate. The results of the fitting procedures are given as the solid lines in Figure 5, and the obtained relative binding constants K'_i/K_i are shown in the figure legend. The magnitude of the decrease in the relaxation rates would seem to indicate that phosphate competes for the high affinity site(s) on both oxidized and reduced cytochrome *c*. The values of the relative binding constants, K'_i/K_i , show that phosphate binds with approximately the same affinity as chloride to the sites observed.

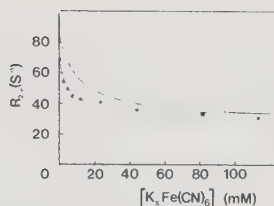


FIGURE 6: The $^{35}\text{Cl}^-$ excess transverse relaxation rate of a 0.054 M NaCl solution, containing Fe^{3+} -cyt *c* (pH 7.3) and Fe^{2+} -cyt *c* (pH 7.4), as a function of the total concentration of added iron hexacyanide. To the oxidized protein (O) ferricyanide ($X = 3$) was added, and to the reduced cyt *c* (●) ferrocyanide ($X = 4$) was added. The data are normalized to a protein concentration of 3.0 mM.

Competition between Iron Hexacyanide and Chloride Ions.

Iron hexacyanide binding to cytochrome *c* is a well-studied phenomenon. NMR measurements by Stellwagen & Shulman (1973) showed that a complex is formed between the two species. Equilibrium dialysis experiments have shown that ferricytochrome *c* contains two iron hexacyanide binding sites, and that chloride ion interacts with both of these binding sites (Stellwagen & Cass, 1975). One of these binding sites is proposed to be located near the exposed heme edge, and ^{13}C NMR of guanidinated cyt *c* suggests the lower portion of the exposed heme edge (Lys-79) is not directly involved in the iron hexacyanide binding (Stellwagen et al., 1977). Due to experimental limitations, Stellwagen & Cass were not able to make a definite statement about the binding parameters for reduced cyt *c*. Figure 6 shows the $^{35}\text{Cl}^-$ relaxation rate in a 0.054 M NaCl solution, containing oxidized or reduced cytochrome *c*, as a function of the total concentration of added iron hexacyanide. To avoid changing the oxidation state of cyt *c*, we added ferrihexacyanide to Fe^{3+} -cyt *c* and ferrocyanide to Fe^{2+} -cyt *c*. The data were obtained by using 3.0 mM oxidized and 2.5 mM reduced cyt *c*, but the data are normalized to a protein concentration of 3.0 mM. The data in Figure 6 suggest that chloride competes with the iron hexacyanides for (a) common site(s) in both oxidation states. The magnitude of the decrease of the relaxation rate implies that the high affinity chloride binding sites are involved in the hexacyanide binding. A rough analysis of the binding curves shows that ferrocyanide competes at least twice as efficiently with chloride for ferrocyanide cytochrome *c* as does ferricyanide in the case of ferricytochrome *c*. Investigations by Margalit & Schejter have pointed out that addition of negatively charged ions such as chloride and phosphate affects the redox potential of cytochrome *c* (Margalit & Schejter, 1970, 1973a,b). The redox potential has been measured indirectly by observing the equilibrium:



Our results suggest an interpretation of this phenomenon. Since chloride ions are shown to compete with iron hexacyanide, we propose that the changes of the redox potential upon addition of chloride result from a simple competitive effect rather than from structural or dynamic changes induced by chloride.

Effect of Cyanide on the $^{35}\text{Cl}^-$ Relaxation Rate. Cyanide ions are known to displace methionine-80 as the sixth iron ligand in ferricytochrome *c* (Wütrich, 1971). Figure 7 shows the effect of the addition of cyanide on the $^{35}\text{Cl}^-$ relaxation rate for cytochrome *c* solutions at neutral and alkaline pH. The upper curve in Figure 7 illustrates the result of addition of KCN to a 0.25 M NaCl solution, pH 7.2, containing 6.0

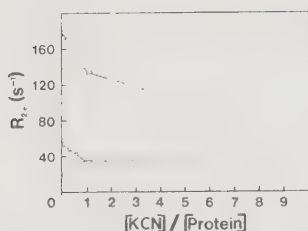


FIGURE 7. The $^{35}\text{Cl}^-$ excess transverse relaxation rate of a 0.25 M NaCl solution, containing 6.0 mM oxidized cytochrome *c* at pH 7.2 (O), and a 0.30 M NaCl solution containing 2.9 mM oxidized cytochrome *c* at pH 9.9 (□), as a function of the cyanide/protein ratio.

mM oxidized cytochrome *c* and shows that cyanide has a marked effect on the chloride binding. In the phosphate and iron hexacyanide competition experiments, we interpreted the decrease in the $^{35}\text{Cl}^-$ relaxation rate as arising from direct competition with the chloride ions. This is a reasonable assumption, since there is no evidence of structural changes upon binding these ions to the protein molecule. Cyanide is known to replace methionine-80 as the sixth heme ligand, with a resulting conformational change (Wütrich, 1971). The data in Figure 7 show a kink near a cyanide/protein ratio = 1, and it is reasonable to assign the first part of this curve to this conformational change. In the second part of the upper curve (i.e., cyanide/protein ratio > 1), the relaxation rate decreases, presumably due to competition with other chloride binding sites. In order to find out what causes the "hump" in the pH dependence of the $^{35}\text{Cl}^-$ relaxation rate in the presence of Fe^{3+} -cyt *c*, we added KCN to a 0.3 M NaCl solution, pH 9.9, containing 2.9 mM oxidized cytochrome *c*. The result of this experiment is shown in the lower curve in Figure 7. It is worth noting that in this pH region it took more than 1 h after each addition of cyanide before equilibrium was reached, in agreement with the findings of George & Tsou (1952).

The results presented above suggest that oxidized cytochrome *c* has (a) chloride binding site(s) at or very near the exposed heme edge. When the pH is raised, the charge arrangement near the heme is changed, resulting in either an increased chloride binding or possibly a change in the $^{35}\text{Cl}^-$ quadrupolar coupling constant.

Sodium Binding to Cytochrome *c*. The binding of Na^+ was studied by using 5 and 12 mM NaCl solutions containing varying amounts of reduced and oxidized protein. Within the error of the measurements, no enhancement of the $^{23}\text{Na}^+$ relaxation rate was observed. From these results, we thus conclude that sodium ions do not bind to oxidized or reduced cytochrome *c*. The only alternative interpretation is that there are sodium ions exchanging too slowly to influence the observed signal. It can be estimated that this would require a k_{off} of about 10^2 s^{-1} , which is not a very likely situation. In this instance, our results are at variance with those of Margoliash and co-workers (1970).

Conclusions

The $^{35}\text{Cl}^-$ NMR data clearly show binding to cytochrome *c* in both oxidized and reduced form. Although a detailed interpretation of the data is not possible at this stage, the results clearly reveal a difference in the anion binding properties at the oxidized and reduced form of cytochrome *c*. No evidence of Na^+ binding to the oxidized or reduced protein was obtained.

Iron hexacyanide and phosphate are shown to compete with chloride for the strong chloride binding sites. The effect of

anions on iron hexacyanide binding, thus, seems to be site specific. These results suggest an interpretation of the changes in redox potential upon addition of chloride and phosphate as arising from a simple competitive effect.

The effect of KCN for oxidized cytochrome *c* at neutral pH implies that the slight conformational change, induced by cyanide binding to the heme iron, reduces the binding of chloride. The "hump" in the relaxation rate accompanying the alkaline isomerization of ferricytochrome *c* implies either that the relaxation effects of an existing site are increased or that a new site is formed.

The competition experiments and pH dependence of the $^{35}\text{Cl}^-$ relaxation rate may be taken to indicate that chloride is bound at the "front side" of the molecule and that the ion binding properties of chloride in this area are different for oxidized and reduced cytochrome *c*. Thus, it is reasonable to suggest that the surface structure or dynamics of the "front side" of the molecule is different in the two oxidation states with possible implications for the redox mechanism of cytochrome *c*.

Acknowledgments

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Figure 1.

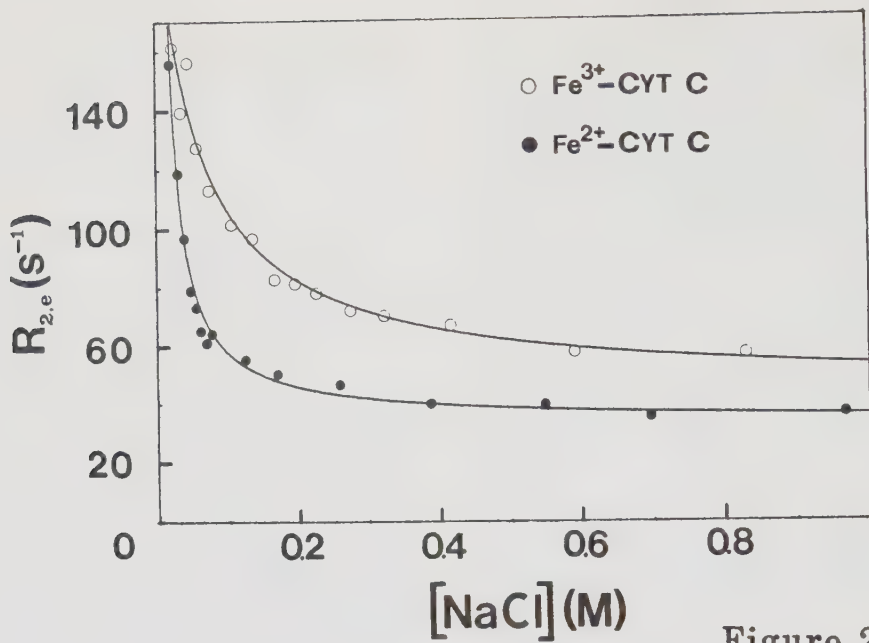


Figure 2.

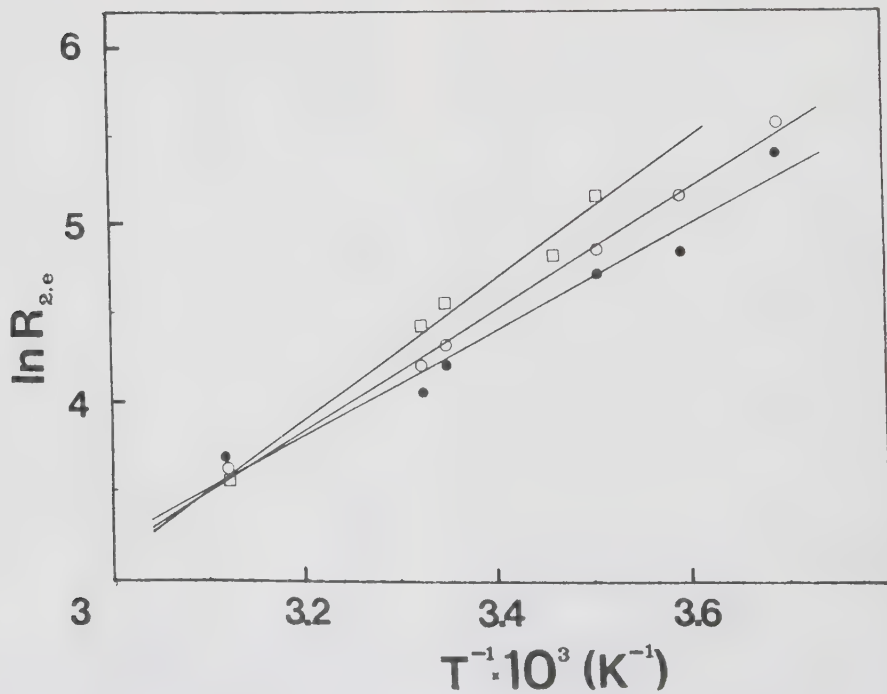


Figure 3.

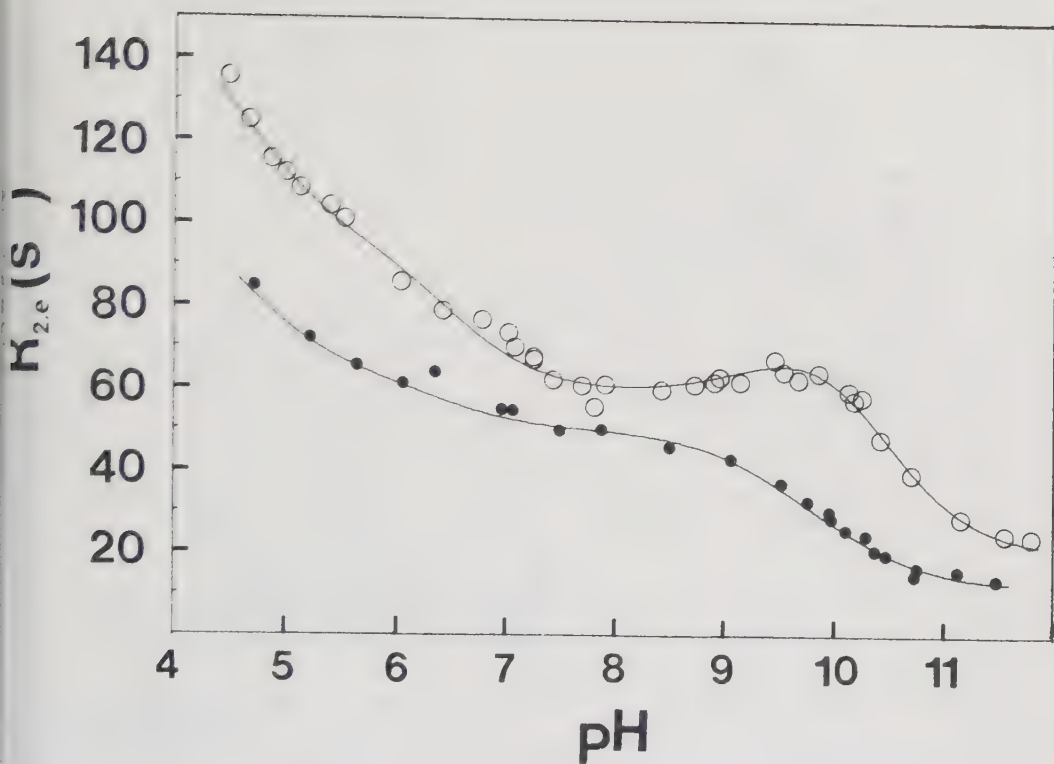


Figure 4.

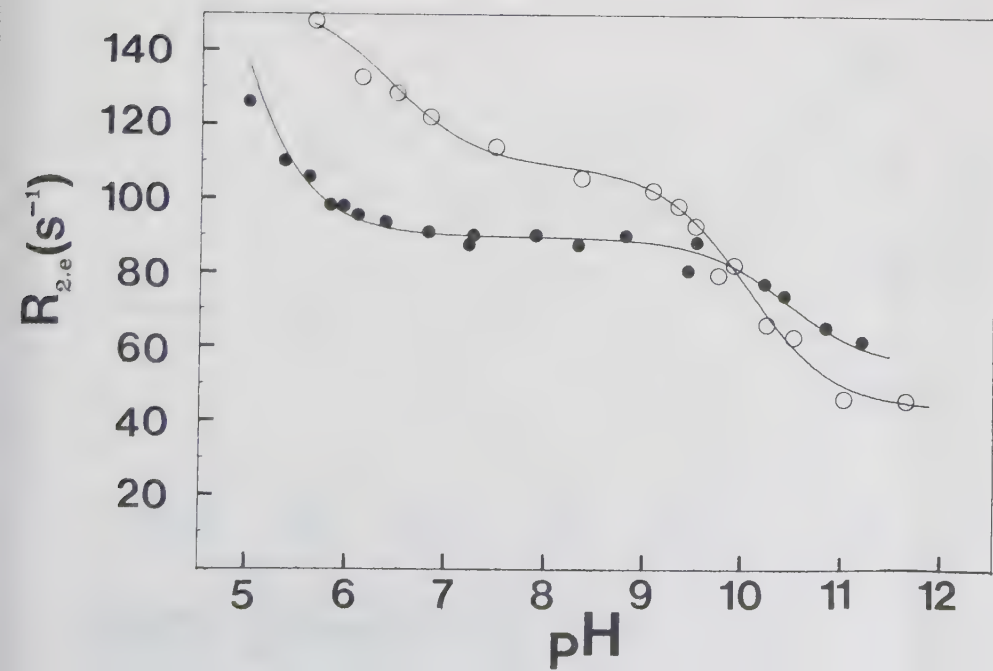


Figure 5.

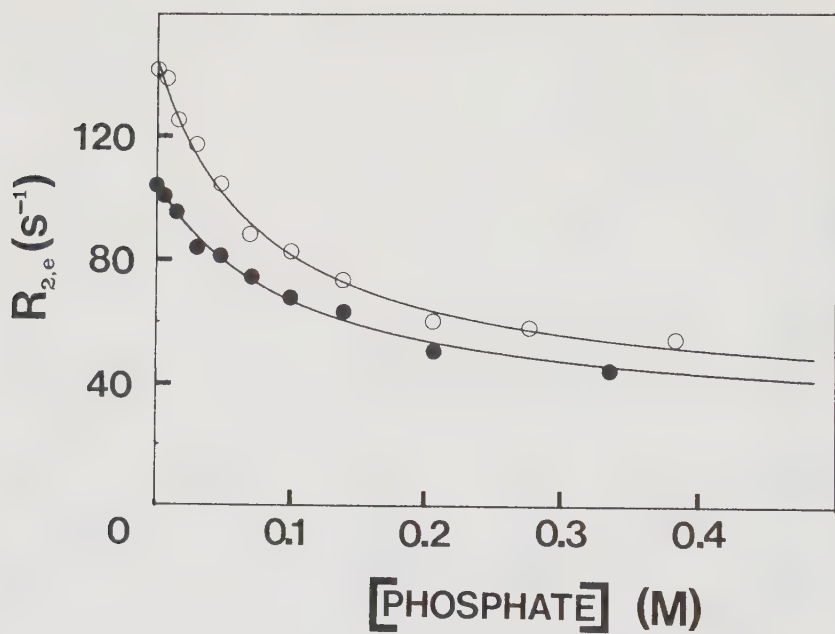


Figure 6.

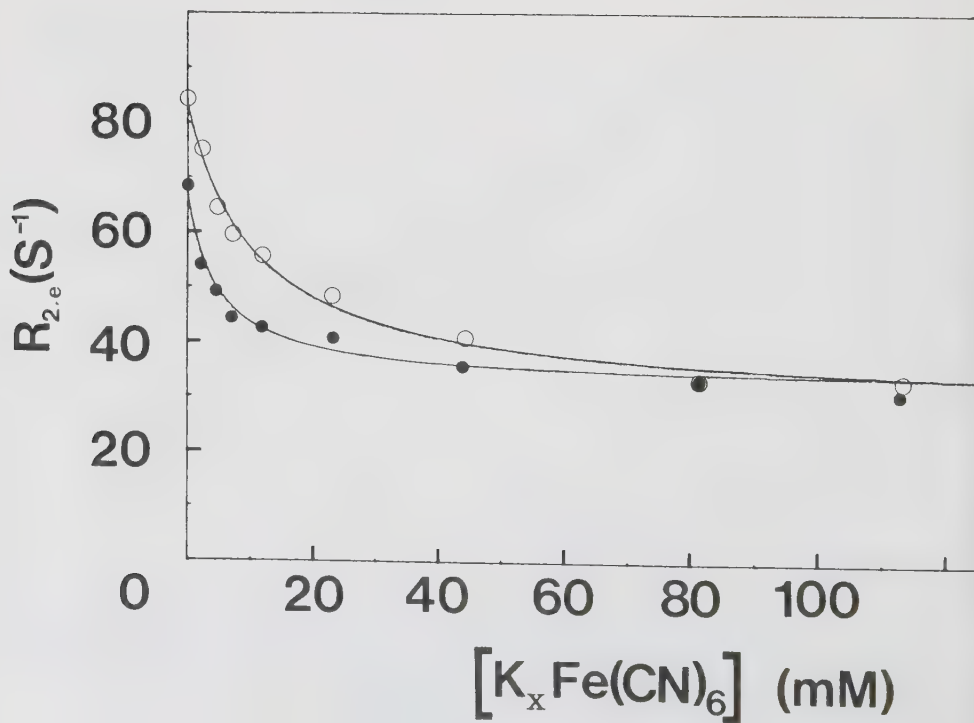
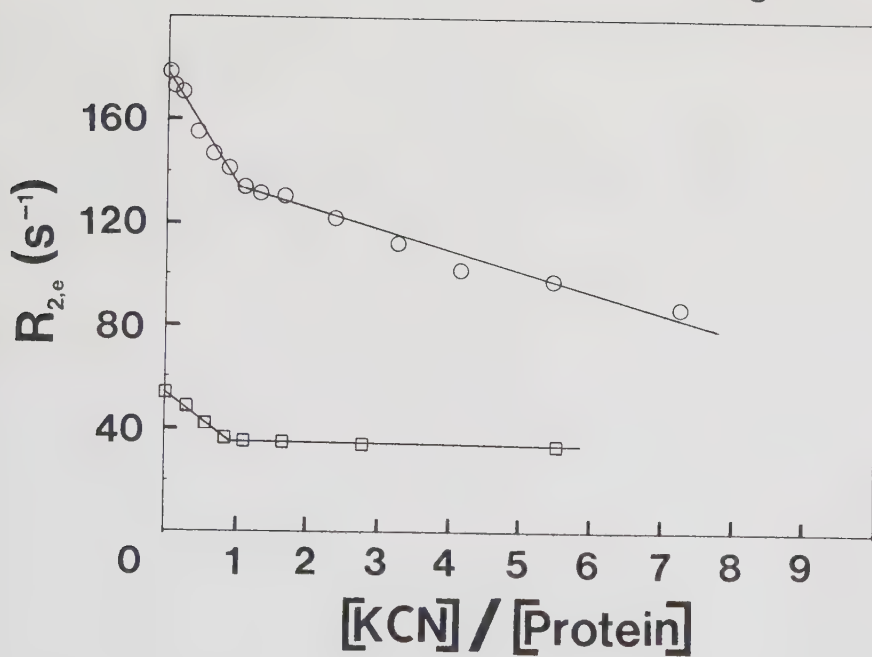


Figure 7.



ARTICLE II

Perchlorate Binding to Cytochrome *c*

A Magnetic and Optical Study

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(Received April 5, 1980)

1. The effects of perchlorate on cytochrome *c* have been investigated by ^1H and ^{35}Cl NMR, electron paramagnetic resonance and optical spectroscopy.

2. The pK values for the formation and disappearance of the major alkaline conformation were found to be displaced from 9.3 to 8.3 and from 10.4 to 10.9, respectively. The displacement was dependent on the ClO_4^- concentration below 0.1 M.

3. Competition experiments between perchlorate and chloride show that ClO_4^- binds both to the neutral and alkaline forms but with a higher affinity for the latter. The appearance of a new binding site in the alkaline form accounts for the markedly enhanced relaxation rate of $^{35}\text{ClO}_4^-$ in this pH range. Complex formation between cyanide and the alkaline species results in the loss of this binding site, which probably is located close to or within the heme crevice.

4. The neutral form of ferricytochrome *c* also binds perchlorate strongly as evidenced by the unique appearance of a high-spin signal dependent on pH and perchlorate concentration. This signal disappears with the same pK value as the neutral form.

The effects of perchlorate on cytochrome *c* are due to specific binding of this ion.

It was recognized early that both oxidised and reduced cytochrome *c* have several distinct pH-dependent conformation states [1–3]. Later investigations have in particular been focused on the state III $\rightarrow$ IV transition of ferricytochrome *c*, a process often referred to as the alkaline isomerization of this protein [4]. A number of spectroscopic methods have been used in order to gain information about how this process is triggered [4–9], and it has been suggested that Met-80, the sixth ligand of the heme iron, is replaced by another strong field ligand, possibly Lys-79 [5].

The pK of the alkaline isomerization ($\text{pH} \approx 9.4$) is susceptible to the presence of anions [10], alcohols [11] and to chemical modifications [12–15] as monitored by the absorption band at 695 nm. This weak band is due to a charge transfer from the sulphur atom of Met-80 to the heme iron [16,17] and it is a sensitive indicator of the native protein.

Studies of the reoxidation kinetics of ferricytochrome *c* [18] showed two kinetic phases each with a pK value of 9.2. In the presence of 0.2 M perchlorate the pK value had shifted to 7.4 but no effects on the pK value of the 695-nm band was observed.

In the present investigation we show that the major effect of perchlorate is a lowering of the pK of the alkaline isomerization as evidenced by several magnetic resonance techniques and optical spectroscopy, and that the interaction with the protein is dependent on the ionization state as well as the oxidation state of the protein.

MATERIALS AND METHODS

Materials

Horse heart cytochrome *c* was prepared according to Brautigan et al. [19]. The protein concentration was determined using an absorption coefficient of $29.5 \text{ mM}^{-1} \text{ cm}^{-1}$ at 550 nm for the reduced protein. Reduction was carried out by addition of ascorbate and excess reductant was removed on a mixed-bed ion-exchange resin. All chemicals were of analytical grade.

Methods

^{35}Cl and ^{37}Cl nuclear magnetic resonance spectra of the perchlorate and chloride ions were recorded at

Abbreviation. EPR, electron paramagnetic resonance.

2.3 T using a modified Varian XL-100-15 spectrometer operating in the Fourier transform mode and at 6.0 T using a home-built Fourier transform spectrometer equipped with an Oxford Instruments wide-bore magnet. The transverse relaxation rate (R_2) was calculated from the linewidth at half height ($\Delta\nu_{1/2}$) of the absorption signal, $R_2 = \pi \cdot \Delta\nu_{1/2}$, with an estimated error of 3%. The longitudinal relaxation rate (R_1) was measured by the inversion recovery method with an estimated error of 5%. All measurements were performed at 28 °C, unless otherwise stated.

^1H NMR spectra of $^2\text{H}_2\text{O}$ solutions of the protein were obtained on a Bruker WH 270 spectrometer. Chemical shifts are quoted as positive downfield relative to 2,2-dimethyl-2-silapentane-5-sulfonate. pH values in $^2\text{H}_2\text{O}$ are the direct meter readings and are designated pH*.

EPR measurements were performed on a Varian E-9 spectrometer operating at 9.12 GHz.

Optical spectra were recorded on a Cary 219 spectrophotometer.

Theory of Quadrupole Relaxation

The ^{35}Cl and ^{37}Cl nuclei have spin quantum numbers (I) of 3/2 and therefore possess electric quadrupole moments (Q), which can couple to electric field gradients at the nucleus. Fluctuations in these field gradients can provide an efficient mechanism for the nuclear magnetic relaxation, so efficient that it in most cases is the only relaxation process that need to be considered [20].

If the chemical exchange of the perchlorate ion between the bulk solution and the protein binding sites is much faster than the relaxation rates at these sites, the longitudinal ($x = 1$) and the transverse ($x = 2$) excess relaxation rate is given by [21]:

$$R_{x,e} = R_{x,0} - R_{x,f} = \sum_i p_i R_{x,i} \quad (1)$$

where $R_{x,0}$ is the observed relaxation rate and $R_{x,f}$ is relaxation rate in the absence of protein, p_i and $R_{x,i}$ are the fraction of ions bound to site i and the relaxation rate at this site, respectively.

For the magnetic field employed in this work 'weakly' non-extreme narrowing conditions ($\omega\tau_c \approx 1$) apply [22] and the intrinsic relaxation rates are given by the equations

$$R_{1,i} = \frac{2\pi^2}{5} \chi_i^2 \left[\frac{0.2\tau_{c,i}}{1 + \omega^2\tau_{c,i}^2} + \frac{0.8\tau_{c,i}}{1 + 4\omega^2\tau_{c,i}^2} \right] \quad (2)$$

$$R_{2,i} = \frac{\pi^2}{5} \chi_i^2 \left[0.6\tau_{c,i} + \frac{\tau_{c,i}}{1 + \omega^2\tau_{c,i}^2} + \frac{0.4\tau_{c,i}}{1 + 4\omega^2\tau_{c,i}^2} \right] \quad (3)$$

where χ_i is the quadrupolar coupling constant for the ion bound to site i and $\tau_{c,i}$ is the correlation time describing the reorientation of the electric field gra-

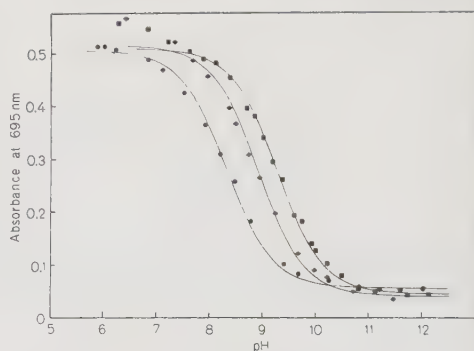


Fig. 1. The absorbance at 695 nm of 0.50 mM ferricytochrome *c* as a function of pH for solutions containing no NaClO_4 (■), 0.018 M NaClO_4 (◆) and 0.19 M NaClO_4 (●). The lines represent the model of a single titration using the following pK values: no NaClO_4 (■) $\text{pK}_a = 9.3$; 0.018 M NaClO_4 (◆) $\text{pK}_a = 8.9$; 0.19 M NaClO_4 (●) $\text{pK}_a = 8.3$.

dients at site i . ω is the resonance frequency in rad/s. By measuring both R_1 and R_2 it is possible to obtain τ_c and χ_i . Eqn (1) shows that in the fast exchange limit there are two main parameters determining the observed relaxation rates, the fraction of bound ions and the intrinsic relaxation rate. The latter is a product of the quadrupole coupling constant and the correlation time according to Eqns (2) and (3).

RESULTS

Effects of Perchlorate on the Alkaline Isomerization of Ferricytochrome *c*

Fig. 1 shows the absorbance at 695 nm of ferricytochrome *c* as a function of pH using varying concentrations of NaClO_4 . The lines represent the results of fitting the experimental data to the equation of a single titration with the corresponding pK values given in the figure legend. It is seen that at neutral pH, and low salt concentrations, the data deviates from this simple model. The pK for the state III $\rightarrow$ IV transition is shifted to lower pH values with increasing amounts of perchlorate, yielding a maximal shift of about 1 at approximately 0.1 M ClO_4^- . This effect was not seen for chloride even at large concentrations (0.5 M).

Fig. 2A shows the pH dependence of the EPR spectrum of ferricytochrome *c* at 15 K in the absence of perchlorate. The spectrum is characterized by a single dominating low-spin form, with g values 3.06, 2.25 and 1.25, at pH values below the alkaline isomerization. As the pH is raised three new low-spin

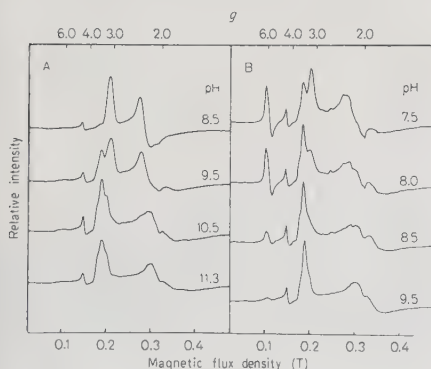


Fig. 2. The pH dependence of the EPR spectrum of ferricytochrome *c*. Spectra were recorded at 9.12 GHz and at 15 K in the absence of NaClO₄ (A) and in the presence of 0.7 M NaClO₄ (B). The protein concentration was 3.0 mM. The spectrometer gain in the top trace in (A) is reduced by a factor of 0.5

species appear. The major form has *g* values around 3.4 and 2.2 (the *g_x* signal is too broad to be detected). The estimated *pK* for the disappearance of the neutral species is 9.4 in agreement with earlier observations [6] and with the value observed for the 695-nm band (Fig. 1).

Addition of perchlorate to ferricytochrome *c* results in the appearance of a high-spin signal at *g* = 6.0 which becomes detectable above 0.1 M. The intensity of this signal increases with increasing amounts of NaClO₄ and reaches approximately 15% of the total signal intensity at 1.0 M NaClO₄ (pH 5.5 and 10 K). At this pH the remaining low-spin signal is considerably broadened. The high-spin signal is pH dependent and shows a *pK* around 8.3 (Fig. 2B). Furthermore, the *pK* value for the low-spin transition has now also been displaced to about 8.3 and the relative amounts of the three low-spin forms have changed. The small signal seen at *g* = 4 represents an artefact due to impurities.

Fig. 3 shows the low-field parts of the ¹H NMR spectrum of ferricytochrome *c* as a function of pH* in the presence of 0.2 M perchlorate.

The disappearance of the native form is conveniently monitored by the intensity of the heme methyls at 31 ppm and 34 ppm, located on pyrrole rings 2 and 4, respectively [23], since the chemical exchange between alkaline and native forms is slow, resulting in two superimposed spectra [5, 7]. The resonances which presumably correspond to the two peaks identified as heme methyls in the neutral form appear at 23.4 ppm and 21.0 ppm in the alkaline form [5, 7]. In the absence of perchlorate a *pK** of approximately 9.0 was obtained as found earlier [5, 7], while in the presence of this ion a *pK** of 8.2 was observed.

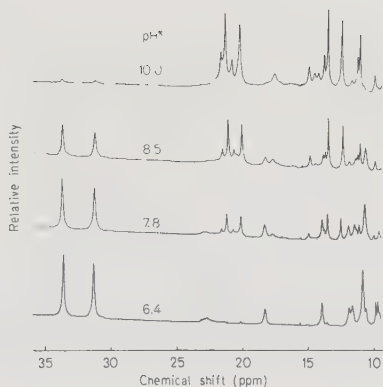


Fig. 3. The pH* dependence of the low-field part of the ¹H NMR spectrum of ferricytochrome *c* in the presence of 0.2 M NaClO₄ (310 K). The protein concentration was 5 mM

The lines of the alkaline form appear with the same *pK** values as for the disappearance of the two signals above, with the only difference that in the presence of perchlorate the lines are shifted approximately 2.5 ppm upfield.

³⁵Cl Relaxation Measurements

The effect of pH on the ³⁵Cl transverse relaxation rate of the ClO₄⁻ ion in the presence of oxidized and reduced cytochrome *c* is shown in Fig. 4. The data were obtained at 2.3 T using 0.19 M NaClO₄ and 3.0 mM ferricytochrome *c* or ferrocyanochrome *c*.

The experimental relaxation rates in Fig. 4 were fitted to the equation:

$$R_{2,c} = \frac{R_2(\text{III})}{1 + 10^{(\text{pH} - \text{pK}_1)}} + \frac{R_2(\text{IV})}{1 + 10^{(\text{pK}_1 - \text{pH})} + 10^{(\text{pH} - \text{pK}_2)}} + \frac{R_2(\text{V})}{1 + 10^{(\text{pK}_2 - \text{pH})}} \quad (4)$$

where *R*₂ (III), *R*₂ (IV) and *R*₂ (V) denotes the relaxation rate of perchlorate ions exchanging with the neutral form and the alkaline forms of cytochrome *c*, respectively. *pK*₁ and *pK*₂ refer to the formation and disappearance of state IV (see Discussion). The result of the fitting procedure is shown in Fig. 4 with the corresponding parameters given in Table 1. Assuming all three forms of ferricytochrome *c* show nearly the same contribution to the relaxation rate of 'non-specifically' bound ions then it can be calculated that the site(s) responsible for the large enhancement in

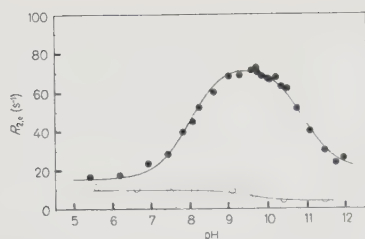


Fig. 4. The ^{35}Cl excess transverse relaxation rate of the ClO_4^- ion as a function of pH for a 0.19 M NaClO_4 solution containing oxidized (●) or reduced (○) cytochrome c. Data are normalized to a protein concentration of 3.0 mM. Lines are theoretical curves using the parameters in Table 1. The measurements were performed at 2.3 T

Table 1. The results of fitting the pH dependence of the ^{35}Cl transverse relaxation rate for the ClO_4^- ion in the presence of ferricytochrome c

Ferricytochrome c state	pK	R_2 s^{-1}
III	8.0	16
IV		75
V	10.9	18

the relaxation rate of state (IV) is approximately 55 s^{-1} . It is worth noting that approximately the same pK value is obtained for the alkaline isomerization of the protein in 0.19 M NaClO_4 using ^1H NMR, EPR and optical spectroscopy (see above).

A similar shape of the relaxation rate curve, produced by the alkaline isomerization, has also been observed for the chloride ion [24] but the pK value of the transition is not shifted towards lower pH values (in accordance with the observations of the 695-nm band under similar conditions). It should also be noted that the pK value for the disappearance of state IV is shifted towards higher pH values in the presence of ClO_4^- , 10.4 [24] to 10.9. Fig. 4 also shows that the relaxation rate in the presence of reduced cytochrome c is slow in the whole pH range with a small transition around pH 9.5, in accordance with the measurements of the ^{35}Cl relaxation of the chloride ion [24]. Reduced cytochrome c remains in a single closed conformation in the pH range.

Since the heme iron in oxidized cytochrome c is paramagnetic the question arises whether the relaxation of the ^{37}Cl and ^{35}Cl nuclei is of quadrupolar or paramagnetic nature. In order to answer this question the transverse relaxation rates of ^{35}Cl and ^{37}Cl for the ClO_4^- ion were measured at pH 9.2 (see below) at 6 T (corresponding to frequencies of 25.0 MHz and 20.8 MHz respectively). If the relaxation results

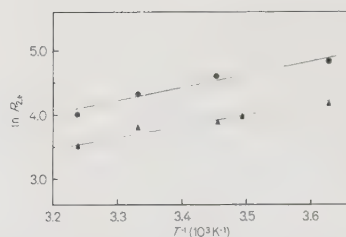


Fig. 5. The temperature dependence of the ^{35}Cl transverse (●) and longitudinal (▲) excess relaxation rates for a 0.18 M NaClO_4 solution, pH 9.2, containing 5.2 mM ferricytochrome c. The data were recorded at 6.0 T

from dipole-dipole interaction, then the ratio $R_{2,\text{ex}}(^{35}\text{Cl})/R_{2,\text{ex}}(^{37}\text{Cl})$ is easily calculated from the Solomon-Bloembergen equations [25]. By inserting the adequate parameters (using $\tau_c = 10^{-12} \text{ s}$, which is dominated by the electronic relaxation time) the ratio becomes 1.44. If, on the other hand, the relaxation is of quadrupolar nature, the ratio is calculated from Eqn (3) by inserting the appropriate parameters, i.e. the quadrupole moments Q of the two nuclei ($Q \approx \chi$), the resonance frequencies (see above) and the correlation time ($\tau_c = 6 \text{ ns}$, see below). This results in a ratio $R_{2,\text{ex}}(^{37}\text{Cl})/R_{2,\text{ex}}(^{35}\text{Cl})$ of 1.60. The experimentally obtained value is 1.60 ± 0.05 , indicating that quadrupole relaxation is the dominating relaxation mechanism.

The temperature dependence of the ^{35}Cl longitudinal and transverse excess relaxation rates of ClO_4^- for a 0.19 M NaClO_4 solution, pH 9.2, containing 5.2 mM ferricytochrome c is shown in Fig. 5. If the chemical exchange between ClO_4^- in the bulk solution and the protein binding sites is fast in comparison with the relaxation rates at the protein binding sites, then the observed relaxation rate may be interpreted in terms of a weighted population average of the intrinsic relaxation rates. When fast-exchange conditions apply a plot of $\ln R_{1,\text{e}}$ and $\ln R_{2,\text{e}}$ vs T^{-1} should yield a linear curve with a positive slope. The data in Fig. 5 indicate that the ClO_4^- exchange is fast, thus confirming the validity of Eqn (1).

The relaxation rates R_1 and R_2 were measured for pH values between 5 and 12 for a 0.18 M NaClO_4 solution containing 2.7 mM ferricytochrome c. The ratio $R_{1,\text{e}}/R_{2,\text{e}}$ was constant, 0.45 ± 0.03 , over the whole pH range. Using Eqns (2) and (3) the correlation time, (τ_c), is calculated to be about 6 ns.

The dependence of the excess transverse relaxation rate of ClO_4^- on the NaClO_4 concentration at pH 9.7 is shown in Fig. 6. The recordings were performed at 2.3 T in the presence of 5.0 mM oxidized cytochrome c. The data may be interpreted in terms of a two-site

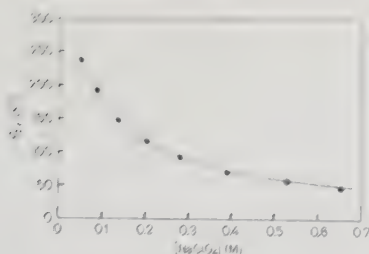


Fig. 6 The ^{35}Cl excess transverse relaxation rate of the ClO_4^- ion as a function of the total NaClO_4 concentration for a 5.0 mM ferricytochrome *c* solution. The data were recorded at 2.3 T. The line represents Eqn (5) using $K_i = 12 \text{ M}^{-1}$, $n_i R_{2,i} = 6.3 \times 10^3 \text{ s}^{-1}$ and $\sum p_w R_{2,w} = 6 \text{ s}^{-1}$.

model having one class of high-affinity sites (index *i*) and one class of low-affinity sites giving a constant contribution ($\sum p_w R_{2,w}$) to the observed relaxation rates. The experimental relaxation rates were fitted to the following equation [24]:

$$R_{2,e} = \frac{n_i c_{pr} K_i R_{2,i}}{1 + K_i [\text{ClO}_4^-]} + \sum_w p_w R_{2,w} \quad (5)$$

where n_i is the number of class *i* sites and K_i is the binding constant at these sites. $R_{2,i}$ represents the relaxation rate of the bound ion. $[\text{ClO}_4^-]$ refers to the concentration of free ClO_4^- and can be approximated by the total NaClO_4 concentration under the experimental conditions used. The total protein concentration is denoted c_{pr} . The results of fitting the experimental data to Eqn (5) appear in Fig. 6 as the solid line and the corresponding parameters are given in the legend. The value of the binding constant ($K_i = 12 \text{ M}^{-1}$) and the correlation time ($\tau_{c,i} = 6 \text{ ns}$, see above) can in combination with Eqns (2) and (3) be used to calculate the upper limit of the quadrupolar coupling constant χ_i (assuming $n_i = 1$). Calculation for different sets of data all give nearly the same value $\chi_i = 0.55 \pm 0.05 \text{ MHz}$.

Competition experiments between chloride and perchlorate were performed in order to find out whether the increased binding of ClO_4^- is specific to this ion. Fig. 7 shows the ^{35}Cl excess transverse relaxation rate of Cl^- for a 0.26 M NaCl solution at (a) pH 9.7 and (b) pH 7.1 as a function of added NaClO_4 in the presence of 1.7 mM oxidized cytochrome *c*. These measurements were recorded at a magnetic field of 2.3 T. The simplest interpretation of the data is that the binding of perchlorate causes a reduction in the number of bound chloride ions. The resulting decrease in the relaxation rate depends on

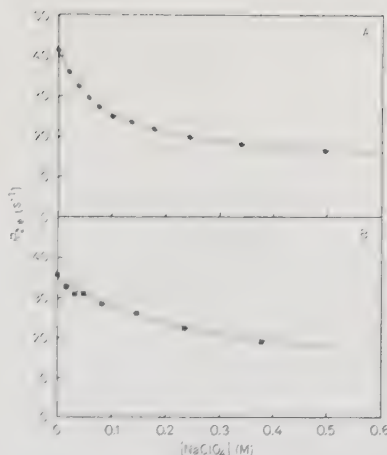


Fig. 7. The effect of NaClO_4 concentration on the ^{35}Cl transverse relaxation rate of the Cl^- ion for a 0.26 M NaCl solution containing 1.7 mM ferricytochrome *c* at (A) pH 9.7 (●) and (B) pH 7.1 (■). The data were recorded at 6 T. The lines are theoretical curves according to Eqn (6) using the following parameters: (A) $n_i R_{2,i} = 29.2 \text{ s}^{-1}$, $K'_i/K_i = 12$, $\sum p_n R_{2,n} = 125 \text{ s}^{-1}$; (B) $n_i R_{2,i} = 27.1 \text{ s}^{-1}$, $K'_i/K_i = 4$, $\sum p_n R_{2,n} = 8.1 \text{ s}^{-1}$.

the amount of added NaClO_4 according to the following expression [24]:

$$R_{2,e} = \frac{n_i R_{2,i} c_{pr} K'_i}{1 + K_i [\text{ClO}_4^-] + K'_i [\text{Cl}^-]} + \sum_{n \neq i} p_n R_{2,n} \quad (6)$$

where n_i is the number of ions bound to site *i*, K'_i and K_i are the intrinsic association constants of Cl^- and ClO_4^- , respectively. $R_{2,i}$ is the intrinsic relaxation rate of the bound chloride ion and c_{pr} refers to the total protein concentration. The concentration of free ClO_4^- and Cl^- are denoted $[\text{ClO}_4^-]$ and $[\text{Cl}^-]$ and, under the experimental conditions used, they may be approximated by the total concentrations of these ions. The second term in Eqn (6) describes the contribution from chloride binding sites which are not affected by addition of perchlorate. The experimental relaxation rates were fitted to Eqn (6) using a least-squares fitting program. The results of the fitting procedures are shown as the solid lines in Fig. 7 and the corresponding parameters are given in the legend. The value of the relative binding constant, K'_i/K_i , indicates that perchlorate binds about 12 times more strongly than chloride to the site observed at alkaline pH and only about 4 times more strongly at neutral pH. By combining the measured binding constant of perchlorate at alkaline pH (see above) with the measured relative binding constant at pH 9.7 the binding constant of chloride at the 'alkaline site(s)' can be calculated to be about 1 M^{-1} . The increase in the

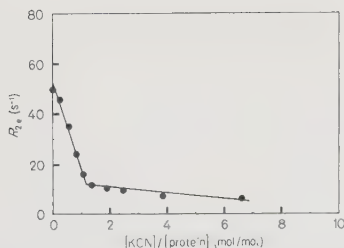


Fig. 8. The effect of the KCN concentration on the ^{35}Cl excess transverse relaxation rate of the ClO_4^- ion for a 1.7 mM ferricytochrome *c* solution at pH 9.7. The measurements were performed at 2.3 T

^{35}Cl relaxation rate of Cl^- due to the alkaline isomerization ($R_{2,1}$) (see [24]) is estimated to be 31 s^{-1} (3.0 mM protein and 0.3 M NaCl). Making use of $K'_1 = 1\text{ M}^{-1}$ and Eqn (5) the relaxation rate of chloride at the binding site(s) is calculated to be approximately $2.6 \times 10^4\text{ s}^{-1}$ (note that only 50% of the protein is in the alkaline conformation, pH 9.5 [6]). By assuming that the correlation time of Cl^- is the same as for ClO_4^- (6 ns) then the upper limit (one binding site is assumed, i.e. $n_i = 1$) of the quadrupole coupling constant of Cl^- bound to the 'alkaline site' is estimated to be 1.1 MHz.

Cyanide is known to displace Met-80 as the sixth heme iron ligand in oxidized cytochrome *c* resulting in a minor conformation change in the protein molecule [26]. Fig. 8 shows the effect of cyanide on the ^{35}Cl excess transverse relaxation rate of ClO_4^- in the presence of 1.7 mM oxidized cytochrome *c* at pH 9.7. It is seen that cyanide effectively reduces the ^{35}Cl relaxation rate up to a cyanide/protein ratio of 1. The decrease in the ^{35}Cl relaxation rate of ClO_4^- following the addition of a given amount of KCN was observed to be a slow process and equilibrium was reached after 0.5–1 h. The same effect of KCN is also seen for Cl^- binding to cytochrome *c* [24].

DISCUSSION

The displacement of the pK value for the alkaline isomerization of oxidized cytochrome *c* by perchlorate is clearly demonstrated by the results presented in Fig. 1–3. The disappearance of the charge-transfer band at 695 nm indicates that the Fe-S bond no longer is intact but does not give any further information about the nature of perchlorate's interaction with the protein.

The EPR experiments show, however, that the interaction between the perchlorate ion and ferricytochrome *c* changes the electronic structure of the heme. The neutral form (state III) of cytochrome *c* reveals the presence of a high-spin signal as well as altered low-spin components. The amount of high-spin form

present at 10 K is about 15%, but the temperature dependence of the signal makes an extrapolation to room temperature difficult. However, the fact that the heme methyls exhibit doubled linewidths at 1.0 M NaClO_4 indicates that a certain amount of high-spin form is present under fast-exchange conditions. The behaviour described above shows close similarities with the pH dependence of the EPR spectrum of cytochrome *c* when Tyr-67 is diiodinated [12].

The interaction between the alkaline form (state IV) of oxidized cytochrome *c* and perchlorate shows a different pattern. The EPR spectrum reveals no high-spin signal and it appears that only the pK values for the major alkaline species have shifted. This is evident from both ^1H NMR and EPR where the intensity of this species in the presence of perchlorate reaches almost the same magnitude as the neutral form at lower pH values. In the absence of perchlorate maximally 50% is obtained [6]. Furthermore, the chemical shifts of the heme methyls are more strongly affected in the alkaline form than in the native form. The data in Fig. 4 show that the ^{35}Cl relaxation rate of ClO_4^- is by far the largest when the protein is in state IV. We may also conclude that the interaction between perchlorate and ferricytochrome *c* depends not only on the conformation of the molecule but also on the oxidation state (Fig. 4). The observation that the shift of the state III $\rightarrow$ IV transition is dependent on the ClO_4^- concentration (Fig. 1), and that a corresponding increase in the pK value of the state IV $\rightarrow$ V transition can be observed (see above), indicates that perchlorate has a higher affinity for state IV and is thus stabilizing this form.

The competition experiments indicate that chloride and perchlorate bind to common sites on ferricytochrome *c* both at neutral and alkaline pH values. The fact that the effect of ClO_4^- binding to state III results in a low enhancement in the relaxation rate of perchlorate may be interpreted in two ways: (a) the chemical exchange of ClO_4^- between the bulk solution and the protein binding site(s) is so slow that the contribution to the observed relaxation rate is (almost) zero [20,21] or (b) the intrinsic relaxation rates at these site(s) are low due to a low 'effective' quadrupole coupling constant. However, our data are not sufficient to discriminate between the two alternatives. The magnitude of the calculated upper limit of the quadrupole coupling constants for both chloride and perchlorate indicate that only a few ions are bound to the site(s) in state IV of oxidized cytochrome *c*.

In the ClO_4^- competition experiments we interpreted the decrease in the ^{35}Cl relaxation rate of Cl^- as arising from direct competition between the two ions. Cyanide is known to displace Met-80 as the sixth heme ligand resulting in a minor conformation change [26]. It is therefore reasonable to assign the decrease in the ^{35}Cl relaxation rate of ClO_4^- produced

upon addition of KCN to this conformation change. The emerging picture of perchlorate binding to cytochrome *c* is as follows: perchlorate is bound to the neutral form of oxidized cytochrome *c*. In the alkaline form of oxidized cytochrome *c* (state IV) a new anion binding site is formed. Binding of perchlorate, as well as other anions (T. Andersson and J. Ångström, unpublished results), stabilizes the structure of this form. It is not possible to assign the anion binding sites on cytochrome *c* at the present stage but our data may be taken to indicate that both chloride and perchlorate ions bind in or near the heme crevice.

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ARTICLE III

CALCIUM BINDING TO PORCINE PANCREATIC PROPHOSPHOLIPASE A₂ STUDIED BY ⁴³Ca NMR

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CALCIUM BINDING TO PORCINE PANCREATIC PROPHOSPHOLIPASE A₂ STUDIED BY ⁴³Ca NMR

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1. Introduction

Pancreatic phospholipase A₂ (PLA₂) catalyzes the hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl *sn*-phospholipids [1]. The enzyme is secreted by the pancreas in an inactive form. In the duodenum the zymogen is activated by trypsin cleavage [1]. The enzyme is capable of degrading phospholipids in micellar form, while the proenzyme is only able to hydrolyze monomeric substrates [2,3]. It has been shown that Ca²⁺ are essential for the catalytic properties of both the enzyme and the zymogen. Ca²⁺ binding to both forms has been studied by a variety of physicochemical techniques, showing that Ca²⁺ binds in a 1:1 molar ratio with association constants in the range of $1-5 \times 10^3 \text{ M}^{-1}$ [4,5]. From X-ray crystallographic data the Ca²⁺ binding site has been found to be located in a cavity of the protein surface near Asp₄₉ [6].

Here we report the results of ⁴³Ca NMR measurements of Ca²⁺ binding to prophospholipase A₂ (PPLA₂). It is shown that the method is useful in providing information about: (i) association constants; (ii) the exchange rate of Ca²⁺ (i.e., k_{off}); (iii) dynamics in the Ca²⁺ binding site; and (iv) the apparent pK value for the group(s) involved in Ca²⁺ binding.

2. Materials and methods

2.1. Materials

Porcine pancreatic phospholipase A₂ was purified from a fortified preparation obtained as a side fraction during insulin production. The chromatographic steps were performed essentially as in [7]. The pro-

phospholipase obtained showed no activity in an egg yolk assay [7]. Protein concentrations were determined spectrophotometrically using the absorption coefficient $E_{1\%}^{1\text{cm}} = 12.3$ at 280 nm [7]. A 0.086 M CaCl₂ solution was prepared by dissolving CaCO₃ (60% isotopically enriched in ⁴³Ca, Oak Ridge Nat. Lab.) in 0.1 M HCl and the solution was neutralized to a final pH at 7.0.

2.2. Methods and data treatment

The ⁴³Ca NMR spectra were obtained at 17.16 MHz, using a homemade Fourier transform spectrometer [8]. The relaxation of ⁴³Ca can safely be assumed to be exclusively quadrupolar. For correlation times sufficiently short, such that $\omega\tau_c \leq 1$, the relaxation can be approximated by a single exponential, corresponding to a Lorentzian line in the Fourier transformed spectrum. When the observed signal is due to metal ions exchanging between two sites, the line shape is no longer Lorentzian, except for very slow and very fast exchange rates. When the intermediate exchange condition applies the full width at half height of the NMR signal, $\Delta\nu$, is not only influenced by the transverse relaxation rates in the two sites and their populations, but also the exchange rate (i.e., k_{off}), cf. the Swift-Connick equation ($x = 1$ indicates longitudinal and $x = 2$ transverse relaxation rates):

$$R_{x,\text{obs}} = (1 - p_b)R_{x,\text{f}} + p_b/(R_{x,\text{b}}^{-1} + k_{\text{off}}^{-1})$$

which is valid for p_b values $\ll 1$ [9]. When the Swift-Connick approximation is not fulfilled, a total band-shape analysis is needed to obtain values of k_{off} and $R_{2,\text{b}}$. The details of the band-shape analysis will be reported elsewhere. To visualize the agreement

between calculated and observed signals, the full width at half height ($\Delta\nu = R_{2,\text{obs}}/\pi$) will be used in the same way as when the Swift-Connick equation is valid.

3. Results

Fig.1 shows the line-width of the observed ^{43}Ca NMR signal as a function of the $[\text{Ca}^{2+}]$ at a constant [protein]. The broadening of the ^{43}Ca signal is most likely due to Ca^{2+} exchanging with the catalytic site of PPLA₂. The data can be fitted to the model of a simple chemical equilibrium:



The details of the fitting procedure will be published elsewhere. The value obtained for the association constant K_a is $2.5 \pm 1 \times 10^3 \text{ M}^{-1}$ at 24°C .

The temperature dependence of the ^{43}Ca NMR line-width is shown in fig.2. By fitting the data we obtained: $k_{\text{off}} = 2.5 \pm 1 \times 10^3 \text{ s}^{-1}$ and the line-width of the bound ion $\Delta\nu_b = 5 \pm 0.6 \times 10^2 \text{ Hz}$ (corresponding to $R_{2,b} = 1.6 \pm 0.2 \times 10^3 \text{ s}^{-1}$) at 24°C . Measurements of the longitudinal relaxation rate, $R_{1,\text{obs}}$, for a 1.7 mM PPLA₂ solution containing 10.0 mM Ca^{2+} (pH 7.0) results in a value of $79 \pm 4 \text{ s}^{-1}$.

The pH dependence of the observed ^{43}Ca NMR line-width is shown in fig.3. The data can be fitted to the model of a single protonation step:

$$\Delta\nu = [\Delta\nu_i] / [1 + 10^{(\text{p}K_{a,i} - \text{pH})}] + C \quad (2)$$

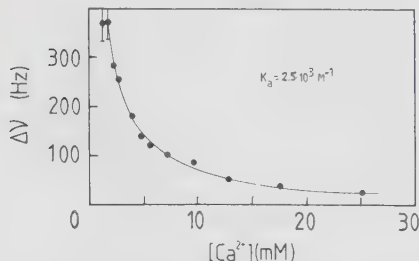


Fig.1. The line-width of the ^{43}Ca NMR signal as a function of the $[\text{Ca}^{2+}]$ for a 2.0 mM PPLA₂ solution (pH 7.5). The spectra were recorded at 24°C . The solid line represents the theoretical curve calculated as described in the text.

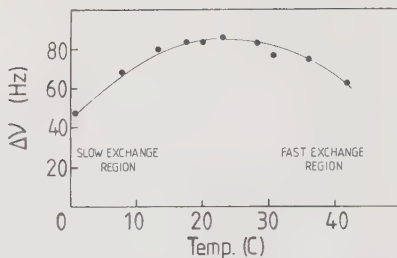


Fig.2. The temperature dependence of the ^{43}Ca NMR line-width for a 1.7 mM PPLA₂ solution (pH 7.4) containing 5.9 mM Ca^{2+} . The solid line is the theoretical curve drawn as described in the text.

In this equation $\Delta\nu_i$ is the step in the line-width associated with the apparent $\text{p}K_{a,i}$. C is the contribution to the observed line-width that stays unaffected during the titration. In the fitting procedure we obtained the following parameters: $\text{p}K_{a,i} = 5.2$, $\Delta\nu_i = 91 \text{ Hz}$ and $C = 8 \text{ Hz}$.

4. Discussion

Ca^{2+} binding to PPLA₂ has been studied using an equilibrium gel filtration technique [4] and UV difference spectrophotometry [4,5]. In these investigations, association constants in the range $2\text{--}4 \times 10^3 \text{ M}^{-1}$ were reported. The value of the association constant obtained from the ^{43}Ca concentration dependence, $K_a = 2.5 \pm 1 \times 10^3 \text{ M}^{-1}$, is in good agreement with these results.

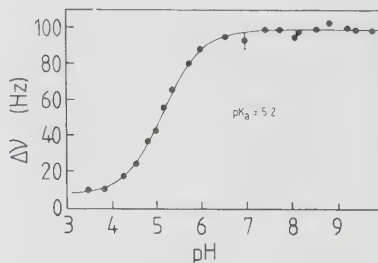


Fig.3. The pH dependence of the ^{43}Ca NMR line-width for a 1.7 mM PPLA₂ solution in the presence of 5.9 mM Ca^{2+} . The spectra were recorded at 23°C . The solid curve represents the model of a single protonation step with an app. $\text{p}K$ of 5.2.

The value obtained for the rate of the dissociation of Ca^{2+} from PPLA_2 (i.e., $k_{\text{off}} = 2.5 \pm 1 \times 10^3 \text{ s}^{-1}$ at 24°C) may be compared with the corresponding figure for rabbit skeletal muscle troponin C (TnC). The off-rate for Ca^{2+} binding to the regulatory sites of TnC ($k_{\text{off}} = 1 \times 10^3 \text{ s}^{-1}$; T. A. et al., unpublished) is of the order of magnitude that one would expect if the on-rate were essentially diffusion controlled. (Taking the association constant of Ca^{2+} to the regulatory sites to be $3 \times 10^5 \text{ M}^{-1}$ [10], we obtain $k_{\text{on}} = 3 \times 10^8 \text{ s}^{-1} \text{ M}^{-1}$.)

In contrast, the on-rate of Ca^{2+} binding to PPLA_2 must be at least two orders of magnitude slower than the on-rates to the regulatory sites of TnC in order to account for the relation between the association constant and the off-rate. This large difference reflects the different nature of the regulatory Ca^{2+} binding sites of TnC on the one hand and that of PPLA_2 on the other. The former sites seem to have a flexible structure, such that the Ca^{2+} binding amino acid residues may easily wrap around an incoming ion. The Ca^{2+} binding site of PPLA_2 seems to be more rigid or generally less accessible to an incoming Ca^{2+} .

The value of the longitudinal relaxation rate, $R_{1,b}$, of the Ca^{2+} at the PPLA_2 binding site may be estimated from the Swift-Connick equation (see above). By using the experimentally obtained values: $R_{1,\text{obs}} = 79 \pm 4 \text{ s}^{-1}$, $R_{1,f} = 0.71 \text{ s}^{-1}$, $p_b = 0.17$ and $k_{\text{off}} = 2.5 \pm 1 \times 10^3 \text{ s}^{-1}$ we may calculate $R_{1,b}$ to be $5.7 \pm 0.8 \times 10^2 \text{ s}^{-1}$.

To obtain an estimate of $R_{2,b}$ we have not followed a procedure similar to that described above for $R_{1,b}$ since chemical exchange effects distort the observed ^{43}Ca NMR signal from a Lorentzian line shape. Instead we have used the value obtained from fitting the variable temperature data: $R_{2,b} = 1.6 \pm 0.2 \times 10^3 \text{ s}^{-1}$. From the ratio $R_{2,b}/R_{1,b}$ it is possible to calculate the correlation time τ_c for the bound Ca^{2+} . Under conditions such that $\omega\tau_c \leq 1$ it may be shown that $[11,12] R_{2,b}/R_{1,b} = (0.3 + 0.5 \tilde{J}_1 + 0.2 \tilde{J}_2)/(0.2 \tilde{J}_1 + 0.8 \tilde{J}_2)$, where $\tilde{J}_1 = (1 + (\omega\tau_c)^2)^{-1}$ and $\tilde{J}_2 = (1 + (2\omega\tau_c)^2)^{-1}$. The quotient $R_{2,b}/R_{1,b} = 2.9 \pm 0.7$ corresponding to $\omega\tau_c = 1.3 \pm 0.3$ or $\tau_c = 12 \pm 3 \text{ ns}$. This value is close to that expected for the rotational diffusion of the entire PPLA_2 molecule. Taking the average hydrodynamic radius to be 2.1 nm this gives $\tau_c = 8 \text{ ns}$ from the Debye-Stokes-Einstein equation [13]. This may be taken to indicate that the Ca^{2+} binding region has a relatively low internal mobility.

From the relaxation rate of the bound $^{43}\text{Ca}^{2+}$

($R_{2,b} = 1.6 \pm 0.2 \times 10^3 \text{ s}^{-1}$) and the correlation time ($\tau_c = 12 \text{ ns}$) we may calculate the quadrupolar coupling constant, χ , of the bound $^{43}\text{Ca}^{2+}$ from the expression [11,12] $R_{2,b} = (2\pi^2\chi^2/49)(0.3\tau_c + 0.5\tau_c\tilde{J}_1 + 0.2\tau_c\tilde{J}_2)$ to be $0.8 \pm 0.1 \text{ MHz}$. This value, which is the first to be reported for a Ca^{2+} binding protein may be compared with the quadrupole coupling constants observed for $^{43}\text{Ca}^{2+}$ bound to EDTA (0.5 MHz) and EGTA (2.1 MHz) (T.D., unpublished).

The apparent pK value ($pK_a = 5.2$) from the pH dependence of the ^{43}Ca line-width probably reflects the ionization of Asp_{49} , the only charged amino acid residue that is directly involved in the Ca^{2+} binding [6]. Our value is in excellent agreement with the value in [14] ($pK_a = 5.2$) for the active enzyme.

Acknowledgement

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ARTICLE IV

A ^{43}Ca NMR AND ^{25}Mg NMR STUDY OF RABBIT SKELETAL MUSCLE TROPONIN C

Exchange rates and binding constants

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1. Introduction

The regulation of vertebrate skeletal muscle contraction involves the binding of Ca^{2+} to a protein complex, troponin–tropomyosin, on the actin thin filament. Troponin consists of three subunits, troponin C (TnC), troponin I and troponin T. The initial event in the contraction is generally considered to be the binding of Ca^{2+} to TnC, which then undergoes a conformational change. In a series of events that are less well documented this conformational change results in the sliding of the thin and thick muscle filaments past each other [1].

TnC has been reported to possess 4 Ca^{2+} binding sites, 2 with $K_a \times 10^7 \text{ M}^{-1}$ (the high affinity sites), and 2 with $K_a \times 10^5 \text{ M}^{-1}$ (the regulatory sites) [2]. Mg^{2+} is also reported to bind to the high affinity sites, with $K_a \times 10^3 \text{ M}^{-1}$ [2]. In addition to the 2 classes of sites described above, there is evidence for an additional class, the weak sites, that bind Mg^{2+} as well as Ca^{2+} with $K_a \sim 10^3 \text{ M}^{-1}$ [2,3].

The contraction and relaxation of skeletal muscles are dynamic processes which are enacted on a millisecond time scale. In the assessment of the roles played in these processes by the different cation binding sites on TnC, the rates of binding and release of Ca^{2+} and Mg^{2+} to and from the different sites are significant parameters. Here, we present a ^{25}Mg and ^{43}Ca NMR study of the binding of Mg^{2+} and Ca^{2+} to TnC, as well as the first direct measurements of the rate of exchange of these ions from the cation–TnC complexes.

2. Materials and methods

2.1. Materials

TnC was prepared from rabbit skeletal muscles according to a slight modification of the procedure in [4]. The purity of the protein was checked on agarose and polyacrylamide gel electrophoreses. In the SDS disc gel electrophoresis one band, corresponding to $\sim 19\,000 M_r$, was obtained. The absorption ratio A_{280}/A_{260} for the protein was 0.89. Ca^{2+} - and Mg^{2+} -free TnC was prepared by slowly passing the protein solution through a Chelex-100 column. The Ca^{2+} content after this treatment was found by atomic absorption spectrophotometry to be $< 0.1 \text{ mol Ca}^{2+}/\text{mol TnC}$. Determinations of the protein concentration were made spectrophotometrically using an absorption coefficient $E_{1\text{cm}}^{1\%} = 0.23 \text{ mg/ml at } 277 \text{ nm}$ [5].

A 0.086 M CaCl_2 solution was prepared by dissolving CaCO_3 (60% isotopically enriched in ^{43}Ca , Oak Ridge Natl. Lab., USA) in 0.1 M HCl. A 0.512 M MgCl_2 solution was made by dissolving MgO (98% isotopically enriched in ^{25}Mg , Oak Ridge Natl. Lab., USA) in 1 M HCl. Both solutions were neutralized to a final pH of 7.0.

2.2. Methods and data treatment

The ^{43}Ca and ^{25}Mg NMR spectra were obtained at 17.16 and 15.61 MHz, respectively, using a home-made Fourier transform spectrometer [6]. The relaxation of both ^{25}Mg and ^{43}Ca can safely be assumed to be exclusively quadrupolar. For correlation times sufficiently short, such that $\omega\tau_c \lesssim 1$, the relaxation can be approximated by a single exponential, corresponding to a Lorentzian line in the Fourier-transformed spectrum. When the observed signal is due to metal ions exchanging between two sites, the line shape is

Abbreviations: TnC, rabbit skeletal muscle troponin C; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulphate

no longer Lorentzian, except for very slow and very fast exchange rates. When the intermediate exchange condition applies the full width at half height of the NMR signal, $\Delta\nu$, is not only influenced by the transverse relaxation rates in the two sites and their populations, but also by the exchange rate (i.e., $k_{\text{off}} = 1/\tau_b$), cf. the Swift-Connick equation:

$$R_{2,\text{obs}} = (1-p_b)R_{2,f} + p_b/(R_{2,b}^{-1} + \tau_b)$$

which is valid for p_b values $\ll 1$ [7]. When the Swift-Connick approximation is not fulfilled, a total bandshape analysis is needed to obtain values of k_{off} and $R_{2,b}$. The details of the bandshape analysis will be reported elsewhere. To visualize the agreement between calculated and observed signals, the full width at half height ($\Delta\nu = R_{2,\text{obs}}/\pi$) will be used in the same way as when the Swift-Connick equation is valid.

3. Results

3.1. ^{43}Ca NMR

The dependence of the ^{43}Ca NMR linewidth on the $[\text{Ca}^{2+}]$ at a constant $[\text{TnC}]$ is shown in fig.1. At high $[\text{Ca}^{2+}]$ this dependence can be simulated assuming that only the regulatory sites have a calcium exchange sufficiently fast to affect the linewidth of the observed

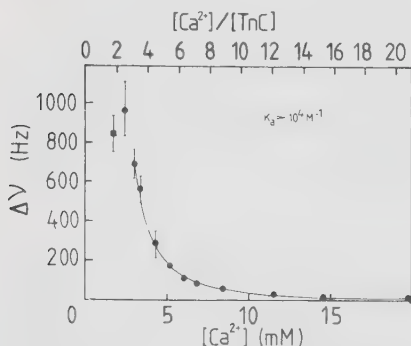


Fig.1. The linewidth of the ^{43}Ca NMR signal as a function of $[\text{Ca}^{2+}]$ for a: (●) 0.94 mM TnC solution at pH 6.6; (■) 1.72 mM TnC solution at pH 7.1, $[\text{Ca}^{2+}] = 3.16$ mM. The spectra were recorded at 23°C. The solid line is a theoretical curve using the parameters obtained from the computer fit described in the text.

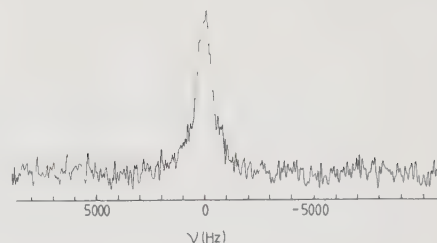


Fig.2. The ^{43}Ca NMR spectrum of a 1.72 mM TnC solution (pH 7.1) containing 3.16 mM Ca^{2+} at 23°C. The spectrum was recorded using a pulse interval of 20 ms and $\sim 2 \times 10^6$ transients where recorded over ~ 12 h accumulation. The signal is ~ 800 Hz broad and is shifted 10 ppm downfield from the signal of free Ca^{2+} .

signal. Using the assumption of two independent sites with the same binding constant we find $K_a^{\text{Ca}} > 10^4 \text{ M}^{-1}$ and $R_{2,b} = 2 \pm 0.2 \times 10^3 \text{ s}^{-1}$ when spectra for $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios from 3–60 are used. The signal seen at a $[\text{Ca}^{2+}]/[\text{TnC}]$ ratio of 1.8 (fig.2) is presumably due to the ^{43}Ca ions bound to the high affinity sites on TnC. The linewidth corresponds to $R_{2,b} = 2 \times 10^3 \text{ s}^{-1}$, which, assuming a correlation time of $1 \times 10^{-8} \text{ s}$, gives a quadrupole coupling constant, $\chi = 0.9 \text{ MHz}$. The temperature dependence of the ^{43}Ca linewidth is shown in fig.3 at two different $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios. Assuming the observed signal to be caused by Ca^{2+} exchanging between the regulatory

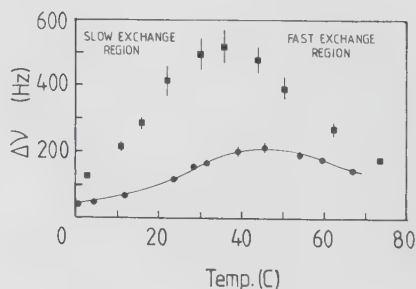


Fig.3. The temperature dependence of the ^{43}Ca NMR linewidth for a: (■) 0.86 mM TnC solution containing 3.67 mM Ca^{2+} at pH 7.0; (●) 0.75 mM TnC solution containing 5.92 mM Ca^{2+} at pH 7.1. The solid line is the theoretical curve drawn using $k_{\text{off}} = 1.0 \times 10^3 \text{ s}^{-1}$ and $R_{2,b} = 2 \times 10^3 \text{ s}^{-1}$.

sites and the bulk solution, with no effect from the high affinity sites, a bandshape analysis results in $k_{\text{off}} = 1.0 \pm 0.1 \times 10^3 \text{ s}^{-1}$ at 23°C . It is also assumed, based on ^1H NMR measurements [8], that there is no large conformational change in the temperature interval used.

When Mg^{2+} is added to a 0.75 mM TnC solution containing 3.0 mM Ca^{2+} , the observed ^{43}Ca NMR linewidth decreases $\sim 60\%$ (20 mM Mg^{2+}). This result may be interpreted in several ways. One explanation is that Mg^{2+} is competing for the same sites as Ca^{2+} . If we assume that the observed ^{43}Ca broadening mainly is due to binding to the regulatory sites this is not a likely explanation since Mg^{2+} is known to bind weakly to these sites [2]. If we assume that the observed ^{43}Ca broadening partly is due to Ca^{2+} binding to the weak sites, what we are observing could be a Mg^{2+} – Ca^{2+} competition for these sites. We consider however, that this a less likely situation since the fraction of ions bound to this class of site(s) is low under the experimental conditions. A remaining explanation is that the decrease in the observed ^{43}Ca NMR linewidth is due to a decrease in the exchange rate of Ca^{2+} to the regulatory sites caused by the binding of Mg^{2+} to the weak sites of TnC. In order to test this the temperature dependence of the ^{43}Ca linewidth was studied (on a 3.0 mM Ca^{2+} solution containing 0.75 mM TnC) in the presence of excess Mg^{2+} (20 mM). This study showed the Ca^{2+} exchange rate to be considerably reduced and that a simple two site exchange model no longer was sufficient to account for the data. A model with two protein sites with exchange rates differing by about a factor of 10 was however found satisfactory.

The pH dependence of the ^{43}Ca NMR linewidth can be described using two pK_a values, 4.6 and 6.0.

3.2. ^{25}Mg NMR

The linewidth of the ^{25}Mg NMR signal as a function of the $[\text{Mg}^{2+}]$ at a constant $[\text{TnC}]$ is shown in fig.4. A bandshape analysis, assuming two sites, results in $K_a^{\text{Mg}} = 5 \pm 1 \times 10^2 \text{ M}^{-1}$ at 23°C .

A bandshape analysis of the temperature dependence of the ^{25}Mg NMR signal gives $k_{\text{off}} = 8 \pm 5 \times 10^3 \text{ s}^{-1}$ and $R_{2,b} = 5 \pm 3 \times 10^4 \text{ s}^{-1}$ at 23°C .

The pH dependence of the ^{25}Mg NMR linewidth can be described using a single pK_a value of 5.4.

Fig.5 shows the effect of added Ca^{2+} on the ^{25}Mg NMR signal. An 80% reduction in the linewidth is observed after the addition of 2 mol Ca^{2+} /mol TnC

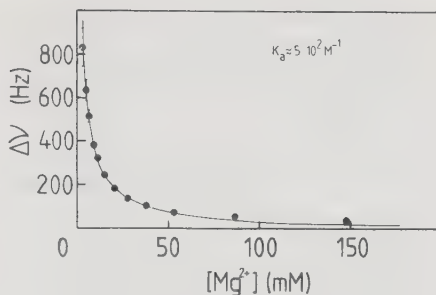


Fig.4. The dependence of the ^{25}Mg NMR linewidth upon addition of Mg^{2+} to apoTnC. The measurements were done at 23°C using a 0.93 mM TnC solution (pH 6.8). The curve represents the results of fitting the data as described in the text.

and after the addition of 4 mol Ca^{2+} /mol TnC $\sim 15\%$ of the initial broadening still remains.

4. Discussion

It has often been stressed that it would be very difficult, if not impossible, to directly observe the NMR signal from a quadrupolar nucleus bound to a protein. We now believe that this was too pessimistic a view, and wish to report what is probably the first NMR signal from a quadrupolar ion bound to a protein:

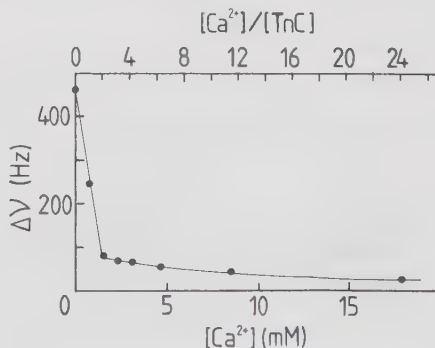


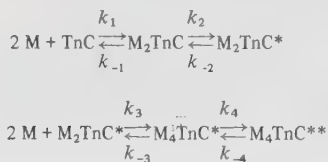
Fig. 5. The ^{25}Mg NMR linewidth as a function of $[\text{Ca}^{2+}]$ for a 2.9 mM Mg^{2+} solution (pH 7.1) in the presence of 0.74 mM TnC. The data were obtained at 24°C .

The ^{43}Ca NMR signal from Ca^{2+} bound to the high affinity sites in TnC (fig.2).

We believe that this signal is due to the protein-bound Ca^{2+} , since the free $[\text{Ca}^{2+}]$ is exceedingly small under the conditions used.

For ^{43}Ca and ^{25}Mg NMR the lower practical concentration limit presently is $\sim 1 \text{ mM}$, which sets an upper limit of $\sim 10^4 \text{ M}^{-1}$ on determinable association constants, K_a . We can therefore not expect a good estimate of K_a for Ca^{2+} to the regulatory sites of TnC, and even less so for the high affinity sites. It is however, encouraging that our analysis of the Ca^{2+} concentration dependence results in a $K_{\text{Ca}} > 10^4 \text{ M}^{-1}$, in agreement with equilibrium dialysis data [2]. For Mg^{2+} binding to the high affinity sites of TnC the conditions are more favourable, with a reported K_a at 4°C of $5 \times 10^3 \text{ M}^{-1}$ [2]. The corresponding ^{25}Mg data, assuming two independent but identical sites, results in $K_a \pm 1 \times 10^2 \text{ M}^{-1}$ at 23°C . This value is an average of all binding sites and at the high concentrations we employed, the weak binding sites can also contribute to the observed line broadening. Fig.5 shows, however, clearly that most of the broadening (80%) is due to the high affinity sites, with a non-negligible broadening also from other weak sites. The shape of the curve at higher $[\text{Ca}^{2+}]$ indicates that Mg^{2+} and Ca^{2+} have approximately the same affinity to these sites.

The kinetics of the metal binding to TnC and the conformational changes caused by the metal binding can be approximated by the following scheme:



The rate of the conformational changes in the protein, steps 2 and 4, have been measured using a stopped-flow technique with a fluorescence probe attached to the protein [9][†]. They found $k_{-2} = 1 \text{ s}^{-1}$ and $k_{-4} = 300 \text{ s}^{-1}$ for Ca^{2+} and $k_{-2} = 8 \text{ s}^{-1}$ and $k_2 = 100 \text{ s}^{-1}$ for Mg^{2+} . The forward reactions of the calcium pro-

tein were too fast to be measured. In [9] it was concluded that the observed structural change (step 4) is the same as the calcium off-rate whereas for step 2 the conformational change is slower than the removal of Ca^{2+} . Under the experimental conditions we have used, the only calcium exchange that can be observed is that involving the regulatory sites. Furthermore, the concentration of the complex Ca_4TnC^* is most probably negligibly small (we are measuring under equilibrium conditions), which means that what we are observing is the exchange rate of calcium in the $\text{TnC}(\text{Ca}_4)$ complex. Our value $k_{\text{off}} = 1 \pm 0.1 \times 10^3 \text{ s}^{-1}$, should be compared to $k_{-4} = 3 \times 10^2 \text{ s}^{-1}$ in [9]. It therefore appears possible that calcium removal from the regulatory sites could be faster than the conformational change.

According to [9] the conformational change following the removal of magnesium from the high affinity sites is slightly faster than that following the removal of calcium, which indicates that the $\text{TnC}(\text{Ca}_2)$ protein conformation is more structured than that of $\text{TnC}(\text{Mg}_2)$, in agreement with the protein NMR study in [8]. However, our data indicate the off-rate of the Mg^{2+} to be 3 orders of magnitude faster than the conformational change. In [2,9] it has been concluded that it is only the Ca^{2+} binding to and release from the regulatory sites that is responsible for the regulation of the muscle activity. While it seems to be quite well demonstrated that the regulatory sites are involved in the regulation of the muscle activity, it seems far less obvious whether or not the high affinity sites are involved.

The temperature dependence of the ^{43}Ca linewidth in the presence of excess Ca^{2+} shows that the Ca^{2+} exchange rate of the two regulatory sites are of equal magnitude (at least within a factor of two). However, in the presence of excess amounts of Mg^{2+} , the Ca^{2+} exchange rates of the two regulatory sites become different (at least by a factor of 10). The Ca^{2+} competition experiments (fig.5) indicate that Mg^{2+} and Ca^{2+} are bound to the same class of low affinity sites, the weak sites, with an equal affinity. Mg^{2+} binding, in contrast to Ca^{2+} binding, to these sites appears to influence the kinetics of Ca^{2+} exchange to at least one of the regulatory sites. The present NMR data thus point to a possible role of Mg^{2+} in regulating Ca^{2+} exchange rates from TnC.

[†] The temperature of the kinetic measurements in [9] is not explicitly given in that article. We will however assume that they were performed at ambient temperature ($23\text{--}25^\circ\text{C}$)

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ARTICLE V

CHARACTERIZATION OF THE Ca^{2+} BINDING SITES OF CALMODULIN FROM BOVINE
TESTIS USING ^{43}Ca and ^{113}Cd NMR

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Running title: Ca^{2+} binding to CaM

INTRODUCTION

The Ca^{2+} ion has long been recognized as an important regulator of a variety of cellular activities [1]. At present it appears that the regulatory effects are mainly due to calmodulin, a low molecular weight protein ($M_r \approx 16700$). The binding of Ca^{2+} to the monomeric protein produces a conformational change which enables calmodulin to associate with and then modulate the activity of enzymes [2].

The amino acid sequence of calmodulin appears to have been highly conserved during evolution and is homologous to those of parvalbumins and troponin C. The protein can be divided into four homologous domains each having a potential Ca^{2+} binding site [3-5]. The binding of Ca^{2+} to calmodulins has been the subject of several studies [6-15]. The majority of these studies show that calmodulin has four Ca^{2+} binding sites but there is some inconsistency about the numbers of high and low affinity sites. ^{113}Cd NMR measurements indicate the presence of two strong sites (the "high affinity" sites) and two weaker sites (the "low affinity" sites) as in the case of troponin C [16-17].

In the present study we use ^{43}Ca and ^{113}Cd NMR in the characterization of the Ca^{2+} binding sites of bovine testis calmodulin (CaM). The use of both ^{113}Cd and ^{43}Ca NMR provides complementary information about the Ca^{2+} sites on CaM.

MATERIALS AND METHODS

Materials

Bovine testis calmodulin was prepared by a modification of the procedure in ref. [16] (the heating steps were not included). The purity of the calmodulin was checked by SDS and agarose gel electrophoresis.

Ca^{2+} free CaM was prepared by passing an aqueous protein solution through a column of Chelex-100. The solution was lyophilized and dissolved in either doubly distilled water (for the ^{43}Ca measurements) or in 10 mM Tris- HClO_4 at pH 7.0. The Ca^{2+} content after this treatment as measured by atomic absorption spectrophotometry was < 0.2 mole Ca^{2+} /mole CaM.

An aqueous 0.115 M CaCl_2 solution was prepared by dissolving CaCO_3 (60 % isotopically enriched in ^{43}Ca , Oak Ridge Natl. Lab., USA) in 0.1 M HCl and then neutralized to a final pH of 7.0. An aqueous CdClO_4 solution was prepared by dissolving CdO (99.5 % isotopically enriched in ^{113}Cd , Oak Ridge Natl. Lab., USA) in 0.25 M HClO_4 . The pH of the solution was then brought back to a final pH of 6.4.

Methods

The ^{43}Ca and ^{113}Cd NMR spectra were recorded at 17.16 and 56.55 MHz respectively using a laboratory-made Fourier transform NMR spectrometer equipped with an Oxford Instruments 6 Tesla wide-bore magnet (room temperature bore: 89 mm). To increase the signal to noise ratio, the probe is constructed with a horizontal sample orientation so that the higher performance solenoid coil arrangement can be used [18]. With this procedure the sensitivity of the instrument is increased by a factor of ~ 2.5 , which corresponds to a gain in time by a factor of ~ 6 . Sample tubes having a volume of 2.6 ml were used. The ^{113}Cd chemical shifts are reported relative to 0.1 M $\text{Cd}(\text{ClO}_4)_2$; shifts to low field are taken as positive values.

Theory

^{43}Ca NMR: The ^{43}Ca nucleus possesses a spin quantum number (I) of $7/2$ and the magnetic relaxation of this nucleus will thus in the general case be a weighted average of 4 exponentials [19]. For sufficiently short correlation times such that $\omega\tau_c < 1.5$ (ω is the angular resonance frequency and τ_c is the correlation time, characterizing the fluctuations in the electric field gradients) the decay of the transverse and longitudinal magnetizations can be approximated by single exponentials [20, 21].

In general it is extremely difficult to observe directly NMR signals from quadrupolar ions bound to proteins [18]. If there is a reasonably fast chemical exchange of ions between the bulk solution and the protein binding sites valuable information about the ion binding can be obtained from the NMR experiments. When the exchange rate, k_{OFF} , is of the same order of magnitude as the relaxation rate of the quadrupolar ion at the protein binding site (intermediate chemical exchange) the shape of the NMR signal is determined by the exchange rate, k_{OFF} , the relaxation rate of the ion on the protein site and the fraction of ions bound to this site. To derive information from the NMR spectrum under these conditions requires a total band-shape analysis. The details of this data treatment will be discussed elsewhere. For ^{43}Ca this type of ion binding study is usually limited to sites having affinities $K_{\text{Ca}} < 10^6 \text{ M}^{-1}$. For larger association constants the exchange rate decrease and the line width of the observed $^{43}\text{Ca}^{2+}$ NMR signal reduces to that of "free" Ca^{2+} ions. Under favourable conditions (i.e. high protein concentrations and very long data accumulation) the ^{43}Ca NMR signals from Ca^{2+} ions bound to relatively small proteins have been observed [21].

^{113}Cd NMR: As discussed above ^{43}Ca NMR is not readily applicable in the study of Ca^{2+} binding to sites having affinities greater than $\sim 10^6 \text{ M}^{-1}$. In the study of this type of site, replacement of the Ca^{2+} ions by $^{113}\text{Cd}^{2+}$ ions has proven useful [16,17]. ^{113}Cd is a spin $\frac{1}{2}$ nucleus and the ^{113}Cd signals from "protein-bound" Cd^{2+} ions are, in contrast to the ^{43}Ca resonances, relatively narrow (20-40 Hz for proteins of moderate size, $M_r = 1-3 \cdot 10^4$). It is worth noting that the ^{113}Cd NMR signals from weaker binding sites can be broadened beyond detection due to chemical exchange effects [16,17].

RESULTS

 ^{43}Ca NMR Measurements

When CaM is added to a CaCl_2 solution at neutral pH the ^{43}Ca NMR signal is considerably broadened. Figure 1 shows the ^{43}Ca NMR line width for a CaCl_2 solution containing 0.93 mM CaM, at pH 7.1. The ^{43}Ca signal observed for a $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio of ~ 2 derives from Ca^{2+} ions bound to the two "high affinity" sites of CaM [21]. This signal is approximately 800 Hz broad and is shifted ~ 10 ppm downfield relative to "free" $^{43}\text{Ca}^{2+}$ ions. When the $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio is increased the observed line width decreases and is displaced towards the resonance position of the "free" ions. In this case ($[\text{Ca}^{2+}]/[\text{CaM}] > 3$) the observed ^{43}Ca signal is an average, exchange-broadened signal due to the binding of Ca^{2+} ions to (a) "low affinity" site(s). A lower limit of the binding constant ($K_{\text{Ca}} > 10^4 \text{ M}^{-1}$) can be estimated from the concentration dependence of the line width.

The temperature dependence of the ^{43}Ca line width for a 4.3 mM CaCl_2 solution, pH 7.0, containing 0.9 mM CaM is shown in Figure 2. At this $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio the observed ^{43}Ca broadening is due to "free" Ca^{2+} ions exchanging with the "low affinity" sites. The temperature profile indicates that intermediate exchange conditions apply. An analysis of the temperature dependence shows the presence of two types of sites with different exchange rates: $k_{\text{OFF}}^{\text{F}} = 1.1 \pm 0.3 \cdot 10^3 \text{ s}^{-1}$ and $k_{\text{OFF}}^{\text{S}} = 20 - 50 \text{ s}^{-1}$ at 25°C .

The pH dependence of the ^{43}Ca NMR line width for a 4.3 mM CaCl_2 solution in the presence of 0.9 mM CaM is shown in Figure 3. The simplest interpretation of the decreased line width between pH 6 and 4 is that the fraction of ions bound to the "weak" site(s) observed is reduced due to protonation of the Ca^{2+} binding carboxylate residues. The data can be described using a single pK_a value of 4.4.

Figure 4 shows the effect of Mg^{2+} on the ^{43}Ca NMR line width for a 2.9 mM CaCl_2 solution, at pH 7.1, in the presence of 0.43 mM CaM. The simplest interpretation of the decreased line broadening is that Mg^{2+} ions compete with Ca^{2+} for the site. An analysis of the data

shows that the affinity for Mg^{2+} is several orders of magnitude smaller than the affinity for Ca^{2+} .

Figure 5 shows the effect of Zn^{2+} on the ^{43}Ca NMR line width for a 2.8 mM CaCl_2 solution, pH 7.0, containing 0.72 mM CaM. At the present time there are several possible interpretations of this effect (see discussion).

^{113}Cd NMR Measurements

Figure 6 shows the ^{113}Cd NMR spectrum of CaM ($[\text{Cd}^{2+}]/[\text{CaM}] = 4$) at different pH values. The two signals at approximately -88 and -108 ppm are attributed to Cd^{2+} ions bound to the "high affinity" sites. The ^{113}Cd signals from ions bound to the "low affinity" sites are broadened beyond detection at neutral pH values due to a relatively slower chemical exchange [16]. The spectra in Figure 6 show that the two "high affinity" signals broaden at low pH values. Analysis of the data shows however that the intensity of the signals remains constant to $\text{pH} \approx 6.2$ and to $\text{pH} \approx 5.3$ for the resonances at -88 ppm and -108 ppm respectively. When the pH is further decreased also the intensities are reduced.

The broadening of the ^{113}Cd signals is most likely due to an increase in the metal ion exchange rate. The decreased intensity of the signals at low pH indicates a reduced metal ion binding to the high affinity sites presumably due to protonation of carboxylate residues. From the pH dependence of the signal intensities it can be estimated that the two "high affinity-signals" have pK_a values differing by a factor of 0.5-1 pH unit.

A broad signal appears at ca. -43 ppm at low pH values. This signal is most likely an average signal from Cd^{2+} ions exchanging between the bulk solution and the "low affinity" sites. At higher pH values this signal broadens further due to a slower chemical exchange of Cd^{2+} .

Figure 7 shows the effect of Zn^{2+} ions on the ^{113}Cd NMR spectrum of CaM. It is apparent from these spectra that the binding of Zn^{2+} increases the exchange rates of Cd^{2+} ions between the bulk solution and the "high

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affinity" and "low affinity" sites of CaM. An integration of the signal amplitudes demonstrates that the intensities of the "high affinity-signals" are reduced with increasing Zn^{2+} concentration.

DISCUSSION

Ca^{2+} binding to CaM has been studied under a variety of experimental conditions [4-12]. Most of these studies indicate that 4 Ca^{2+} ions are bound to each CaM molecule. The values of the binding constants derived from these studies show a considerable scatter. There is no clearcut correlation between the experimental conditions employed and the resulting binding parameters. These studies are mostly based on equilibrium dialysis techniques and the data were analysed by linearized plots. This type of data treatment has been criticized as inaccurate [22].

The ^{113}Cd NMR measurements in ref. 16 and in this work indicate the presence of two sites with high affinity and two sites with a somewhat lower affinity, similar to the case of troponin C [17].

The temperature dependence of the ^{43}Ca NMR signal in Figure 2 shows that the two "low affinity" sites have exchange rates differing by a factor of about 40 at room temperature ($k_{\text{OFF}}^{\text{F}} = 1.1 \pm 0.3 \cdot 10^3 \text{ s}^{-1}$ and $k_{\text{OFF}}^{\text{S}} = 20 - 50 \text{ s}^{-1}$ at 23°C). At low temperatures (30°C and lower), only the site with the faster exchange is probed by ^{43}Ca NMR.

Due to the lower practical concentration limit for Ca^{2+} ($\approx 1 \text{ mM}$) in the present study, binding constants larger than $\approx 10^4 \text{ M}^{-1}$ are not determinable. It should be noted that the association constant derived from the Ca^{2+} concentration dependence in Figure 1, $K_{\text{Ca}} > 10^4 \text{ M}^{-1}$, is in agreement with other binding studies [6-15].

An analysis of the pH dependence of the ^{43}Ca NMR line width in Figure 3 gives an apparent pK value of 4.4. This pK_{a} value essentially describes the competition between H^{+} and Ca^{2+} ions for the "low affinity" site with the faster metal ion exchange (unless the relative magnitudes of the exchange rates for the two "low affinity" sites are markedly changed at lower pH). The pH dependence of the ^{113}Cd spectrum (Figure 6) indicates that the two "high affinity" sites have pK_{a} values differing by ≈ 0.5 -1 pH unit.

The increased exchange rate of Cd^{2+} , produced by Zn^{2+} (Figure 7), is accompanied by a reduction in the signal intensities. This decrease is probably due to competition between Zn^{2+} and Cd^{2+} for the "high affinity" sites. The Ca^{2+} ions released from the "high affinity" sites will reduce the line width of the ^{43}Ca signal. If it is assumed that all Ca^{2+} ions bound to these sites are replaced by Zn^{2+} the line width should be reduced much more than observed (Figure 5, dotted line). If, however, also the exchange rate of Ca^{2+} to the "weak sites" is simultaneously increased this would broaden the ^{43}Ca signal. The gentle slope of the data points in Figure 5 may consequently be due to these two opposing effects. It should be noted that addition of large amounts of Mg^{2+} does not broaden the ^{113}Cd signals.

At the present time we have no certain interpretation of the increased metal ion exchange rates produced by Zn^{2+} and H^+ ions. The increased exchange rates, k_{OFF} , may be taken to indicate that the affinities for divalent metal ions are reduced (k_{ON} is assumed to be diffusion-controlled). This can be due to the binding of cations to amino acid residues at or near the Ca^{2+} binding sites of CaM. It may be noted that Zn^{2+} ions form reasonably strong complexes ($K_a \approx 10^4 \text{ M}^{-1}$) even with simple amino acids [23] and in this respect differ from Mg^{2+} and Ca^{2+} .

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FIGURE LEGENDS

Figure 1. The Ca^{2+} concentration dependence of the ^{43}Ca NMR line width for a 0.93 mM CaM solution. Temp.: 23°C and pH: 7.1.

Figure 2. The temperature dependence of the ^{43}Ca NMR line width for a 4.3 mM CaCl_2 solution containing 0.89 mM CaM, pH: 7.0.

Figure 3. The pH dependence of the ^{43}Ca NMR line width for a 4.3 mM CaCl_2 solution containing 0.89 mM CaM, temp.: 23°C .

Figure 4. The effect of Mg^{2+} on the ^{43}Ca NMR line width for a 3.0 mM CaCl_2 solution containing 0.43 mM CaM, pH: 7.1 and temp.: 23°C .

Figure 5. The effect of Zn^{2+} on the ^{43}Ca NMR line width for a 2.9 mM CaCl_2 solution containing 0.72 mM CaM, pH: 7.0 and temp.: 23°C .

Figure 6. The pH dependence of the ^{113}Cd NMR spectrum of CaM ($[\text{Cd}^{2+}]/[\text{CaM}] = 4$; 1.5 mM CaM and 6.0 mM $\text{Cd}(\text{ClO}_4)_2$). Temp.: 23°C .

Figure 7. The effect of addition of Zn^{2+} on the ^{113}Cd NMR spectrum of CaM ($[\text{Cd}^{2+}]/[\text{CaM}] = 4$; 1.5 mM CaM and 6.0 mM Cd^{2+}). Temp.: 23°C and pH: 7.5

Figure 1

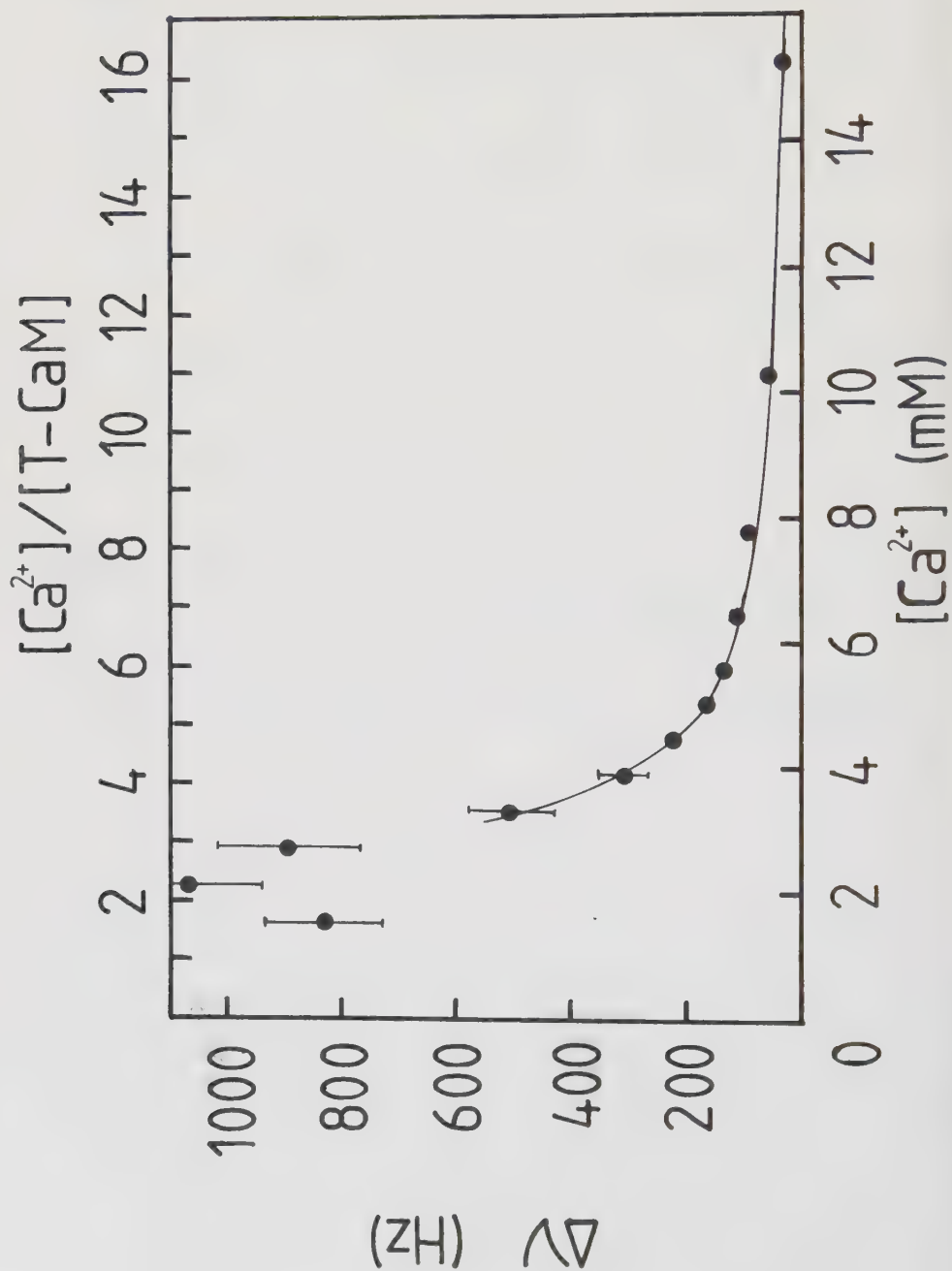


Figure 2.

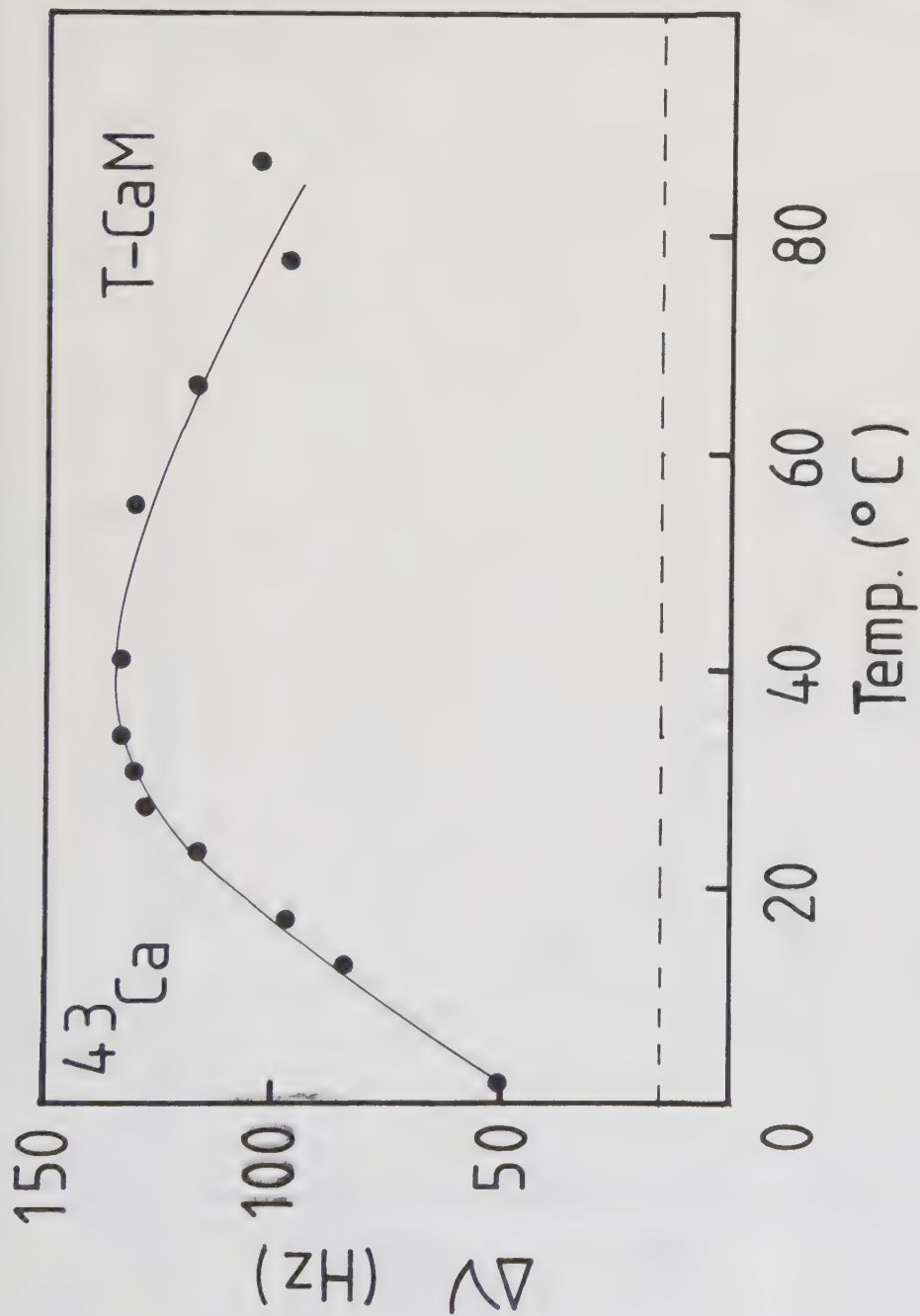


Figure 3.

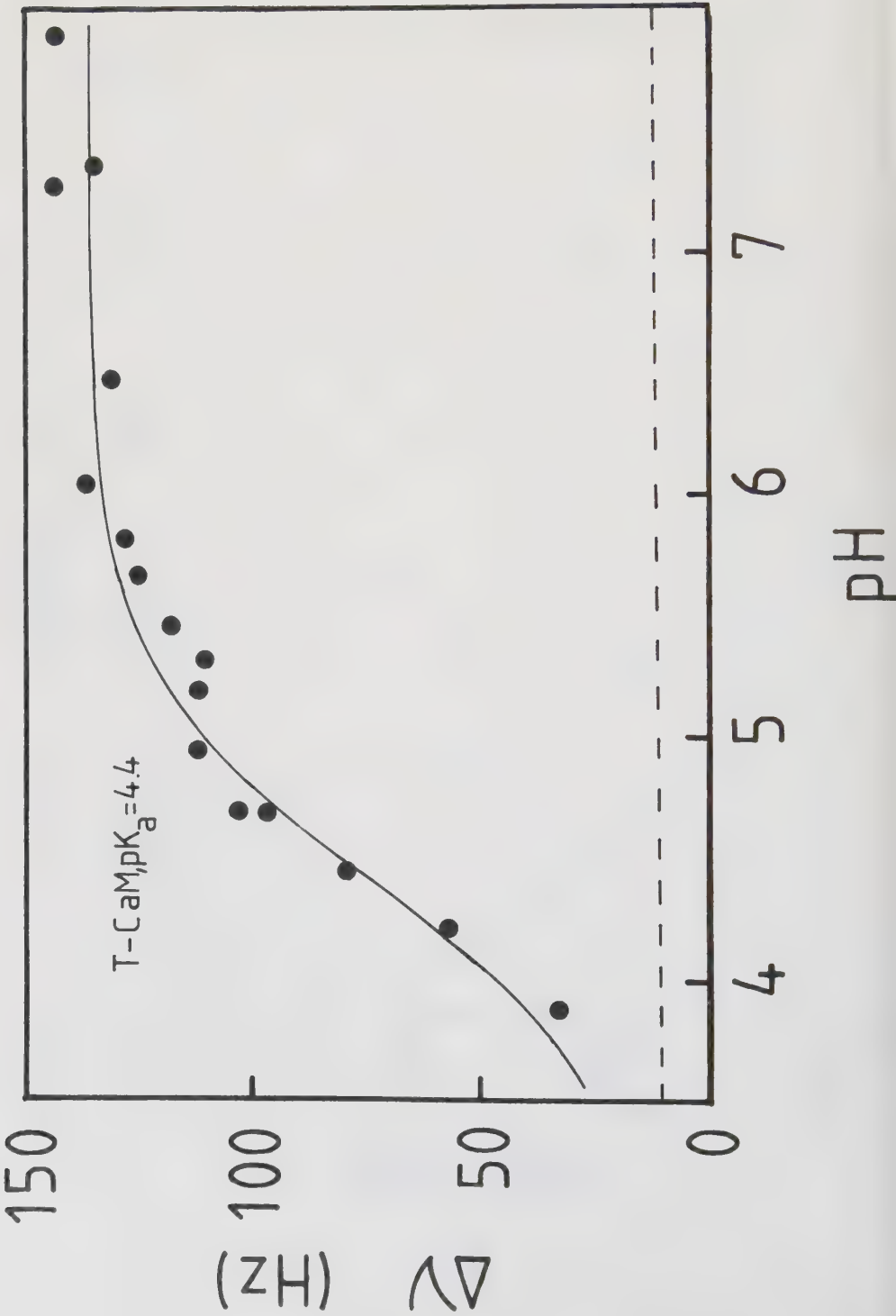


Figure 4.

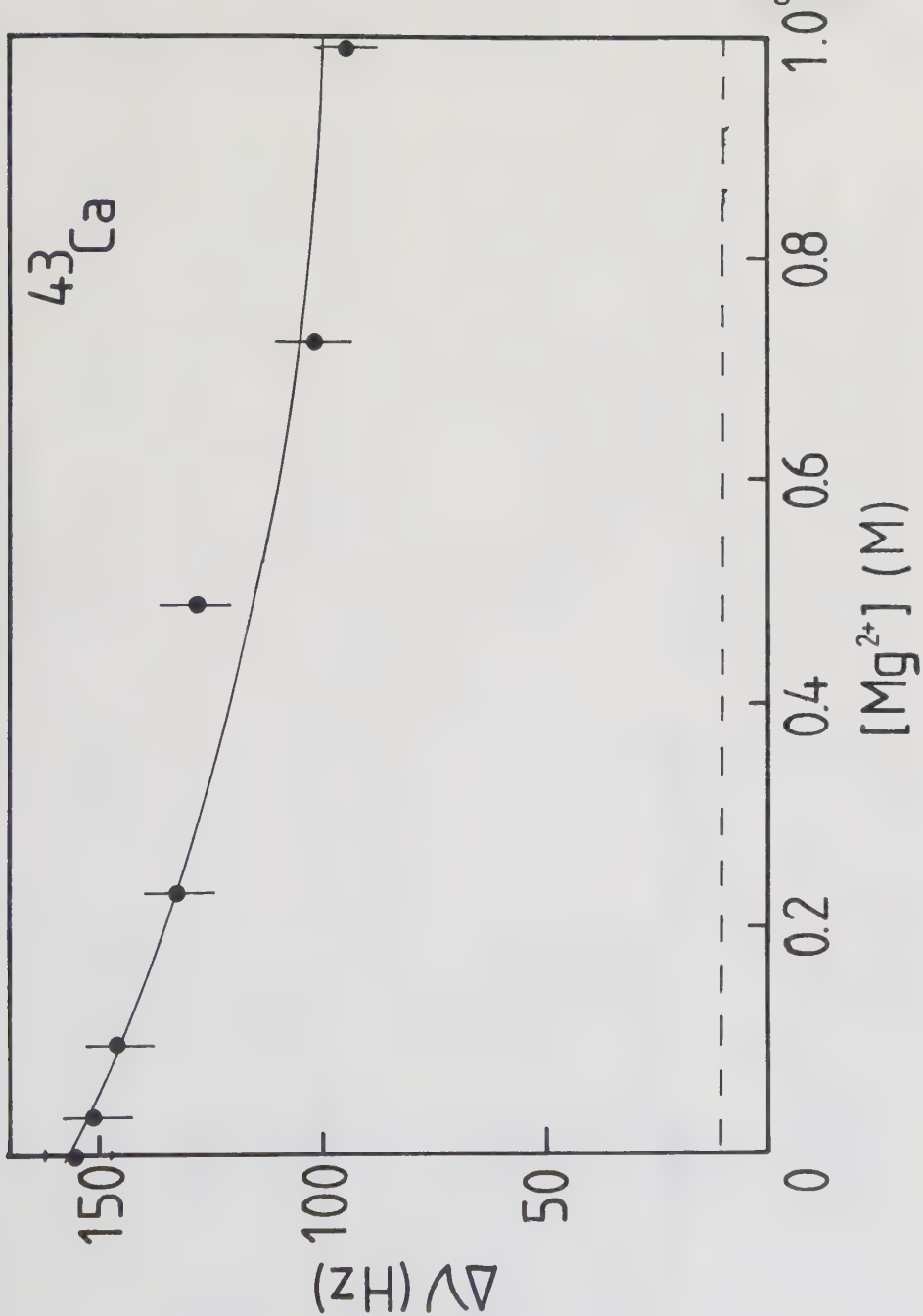


Figure 5.

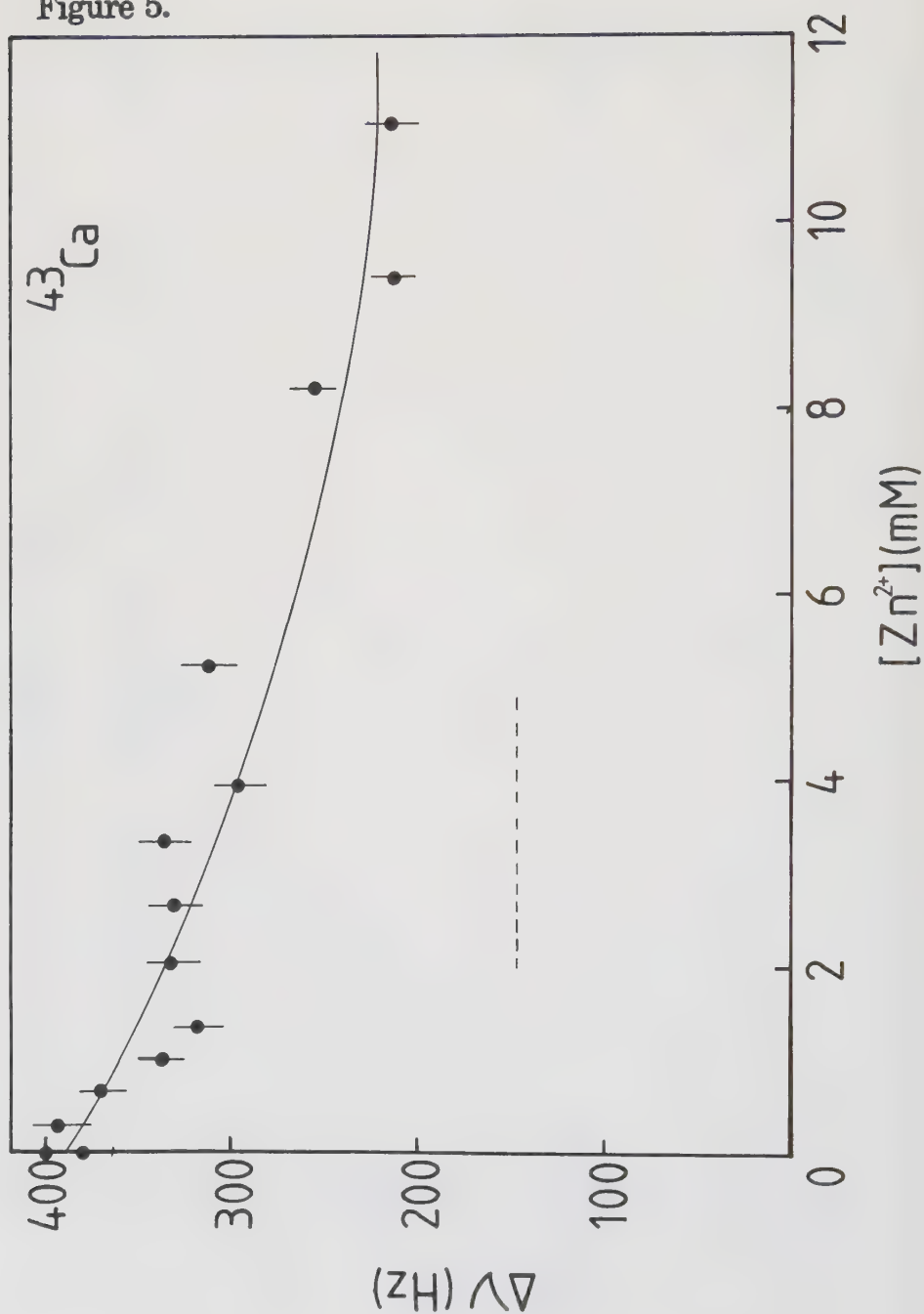


Figure 6.

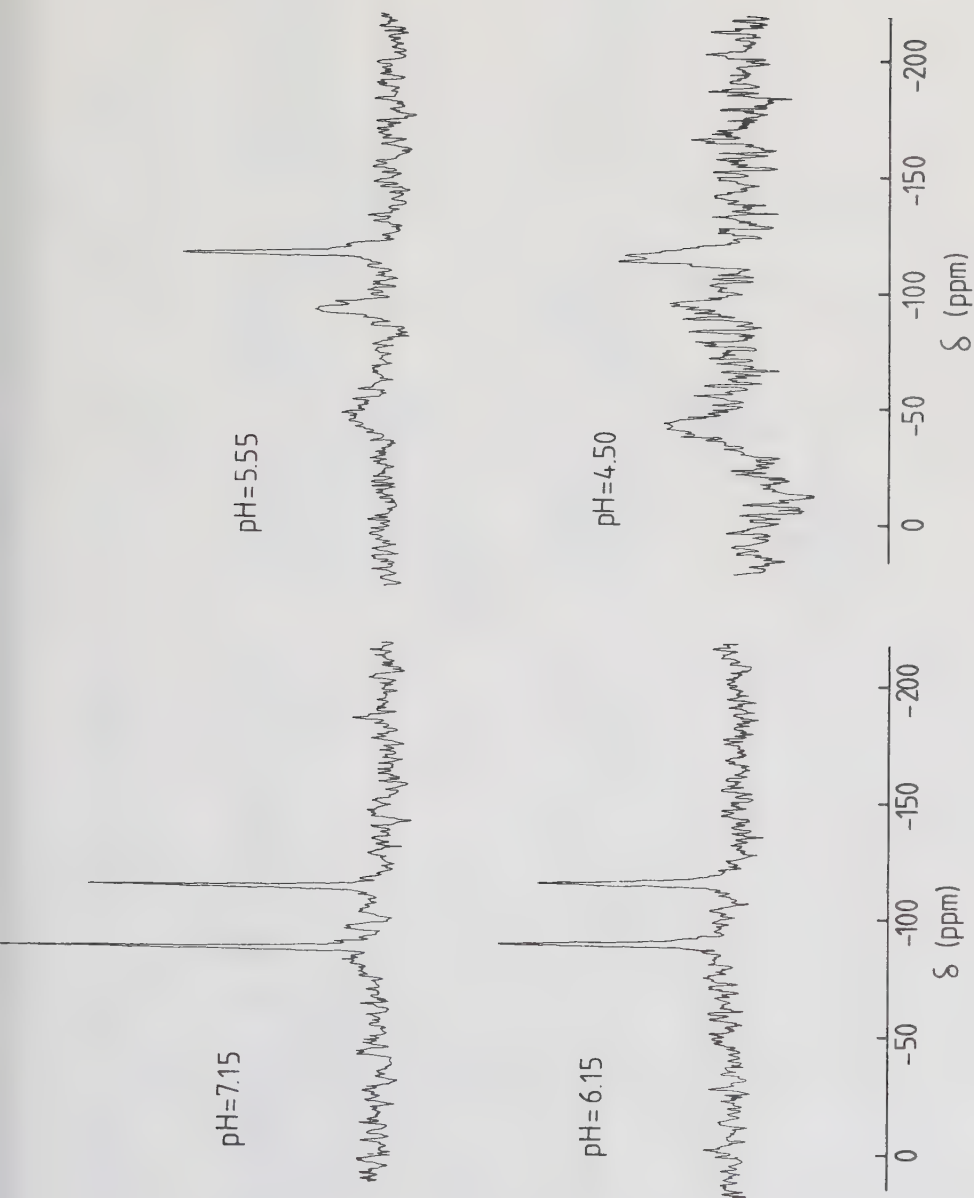
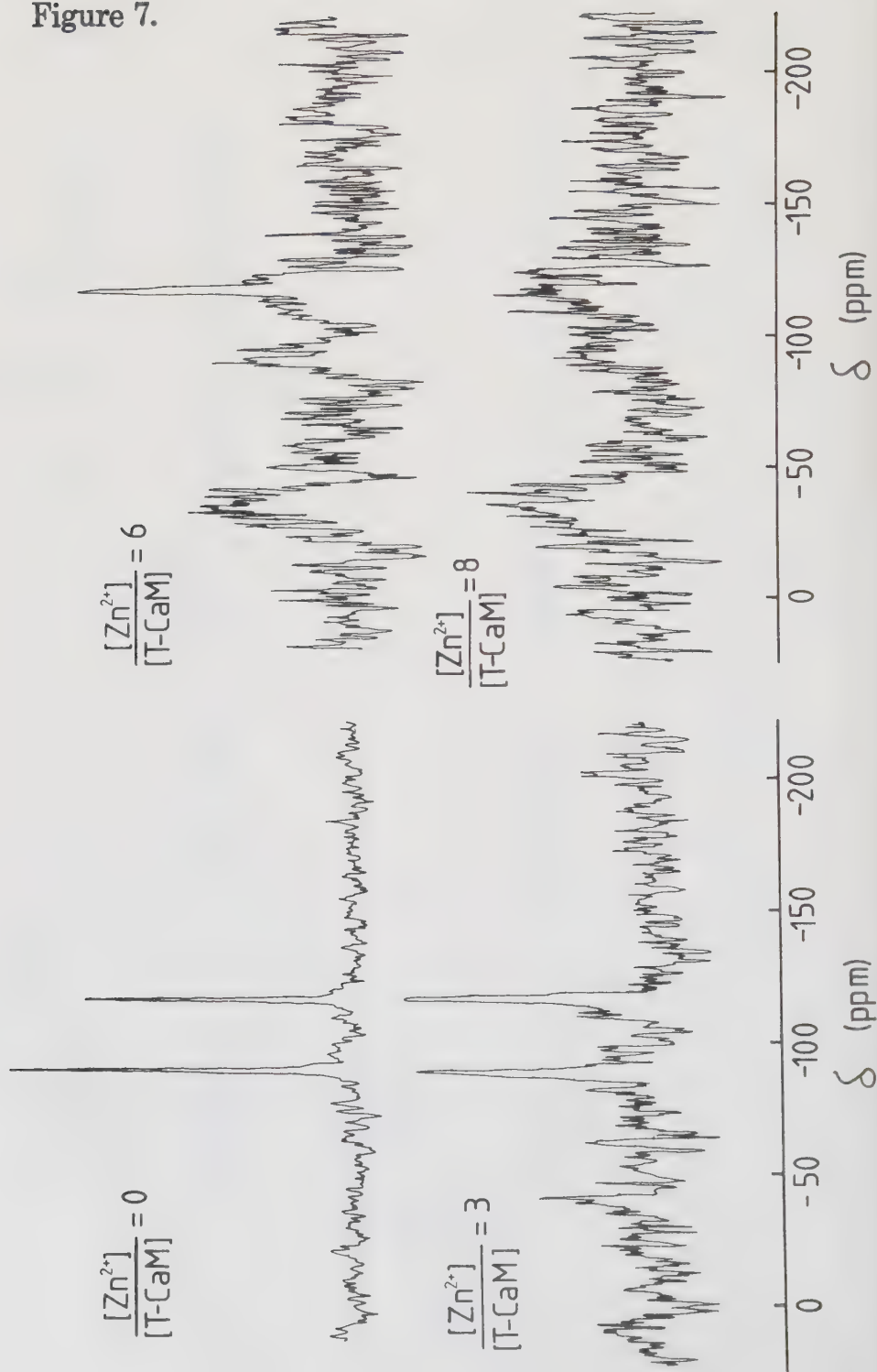


Figure 7.



ARTICLE VI

Direct Observation of ^{43}Ca NMR Signals from Ca^{2+} Ions
Bound to Proteins

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Running title: ^{43}Ca NMR of protein-bound Ca^{2+} .

ABSTRACT

^{43}Ca NMR signals from Ca^{2+} ions bound to the Ca^{2+} binding proteins parvalbumin, troponin C and calmodulin were studied. The observation was made possible by the combined use of isotopically enriched $^{43}\text{Ca}^{2+}$, FT techniques, high magnetic fields and a solenoid type probe design. Measurements of the apparent longitudinal relaxation rate, R_1 , and the transverse relaxation rate, R_2 , provide values for the quadrupole coupling constant and the correlation time. The magnitudes of the calculated correlation times are in good agreement with the rotational correlation time for the entire protein molecule, indicating that the Ca^{2+} binding sites have a comparatively rigid structure.

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INTRODUCTION

Much of the pioneering work on the NMR of the ^{43}Ca nucleus created undue pessimism about the usefulness of this method for the study of Ca^{2+} -macromolecule interactions¹. Many of the earlier investigations were made at a time when Fourier-transform NMR spectrometers and cryomagnets were not available. During the last few years several studies have shown that ^{43}Ca NMR can give interesting information about Ca^{2+} binding to proteins even at millimolar concentrations^{2,3}. These studies were performed on systems with relatively fast chemical exchange of the Ca^{2+} ion between the free and protein bound states. The observed ^{43}Ca signal is then an average signal sensitive to the exchange rate. The application of this method is limited to proteins with Ca^{2+} affinities less than ca. 10^5 - 10^6 M^{-1} .

In the work presented here we show that direct observation of the ^{43}Ca NMR signal of the protein-bound Ca^{2+} ions is possible in suitable cases and that relevant physical and biological information can be obtained from such studies. We have used this approach to study the three Ca^{2+} binding proteins: carp muscle parvalbumin ($\text{pI}=4.25$), rabbit skeletal muscle troponin C and bovine testis calmodulin.

EXPERIMENTAL SECTION

Bovine testis calmodulin (CaM) and rabbit skeletal muscle tropoinin C (TnC) were prepared as described in ref. 4 and ref. 3, respectively. Carp muscle parvalbumin component pI 4.25 (PA) was generously supplied by Prof. J. Parello, Montpellier.

Ca^{2+} -free TnC and CaM were prepared by passing an aqueous solution of the proteins through a column of Chelex-100. The solutions were then lyophilized, and at the time of use dissolved in doubly distilled water. The Ca^{2+} content of TnC and CaM after this treatment, as measured by atomic absorption spectrophotometry, was ≤ 0.2 mol Ca^{2+} per mol protein. For PA, this procedure did not give a sufficient degree of deionization due to the higher affinity of PA for Ca^{2+} . Consequently, Ca^{2+} was replaced by Cd^{2+} ions by simply adding an excess of Cd^{2+} ions to the protein (PA binds Cd^{2+} more strongly than Ca^{2+}), and the excess salt was removed by dialysis. The protein solution was then deionized on the Chelex-100 column, which has a much higher affinity for Cd^{2+} ions than for Ca^{2+} ions, resulting in ~ 0.2 mole Cd^{2+} per mole protein.

An aqueous 0.115 M CaCl_2 solution was prepared by dissolving CaCO_3 (60% isotopically enriched in ^{43}Ca , Oak Ridge Nat.Lab., Oak Ridge) in 1 M HCl. The solution was neutralized to a final pH of 7.0 by adding 1 M NaOH.

The ^{43}Ca NMR spectra were recorded at 17.16 MHz using a laboratory-made Fourier transform spectrometer equipped with an Oxford Instruments 6 Tesla wide-bore magnet (room temp. bore 89 mm). In order to increase the signal to noise ratio the probe is constructed with a horizontal sample orientation so that the higher performance solenoid coil can be used⁵. In this way the signal to noise ratio was found to increase by a factor of 2.5 (corresponding to a gain in time by a factor of 6) compared to the conventional vertical Helmholtz coil design. The length for 90° pulse varied between 50 to 100 μs of an aquisition delay time (dead time) of 300 μs was used in order to have a non observable effect from the ring down in the probe after 10^6 transients.

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The longitudinal relaxation rate, R_1 , was determined by the inversion recovery technique⁶ and the transverse relaxation rate, R_2 , was obtained from the line width at half-height, $\Delta\nu_{\frac{1}{2}}$, using the relation $R_2 = \pi \Delta\nu_{\frac{1}{2}}$.

THEORETICAL SECTION

The magnetic relaxation of a nucleus of spin $I > 1$ is, in most cases, due to the interaction of the nuclear electric quadrupole moment with fluctuating electric field gradients at the position of the nucleus. The relaxation of a nucleus of spin $I > 1$ is, in general, not a simple exponential decay⁷. The decay of the longitudinal ($\alpha = 1$) and transverse ($\alpha = 2$) magnetization for a $I = 7/2$ nucleus is a weighted sum of 4 exponentials:

$$M_1(t) = M_1(\infty) \left[1 - K \sum_{i=1}^4 C_{1,i} \exp(-R_{1,i}t) \right] \quad (1)$$

$$M_2(t) = M_2(0) \left[\sum_{i=1}^4 C_{2,i} \exp(-R_{2,i}t) \right] \quad (2)$$

where $\sum_{i=1}^4 C_{\alpha,i} = 1$ and $K = 2$ for the inversion recovery experiment.

It is not possible to obtain analytical solutions of the relaxation equations, but they can be solved numerically for each $\omega\tau_c$ value⁸ (ω is the resonance frequency in radian/s and τ_c is the correlation time describing the reorientation of the electric field gradients at the nucleus). A single-exponential decay is approached as the relaxation rates of the different components become increasingly alike and/or as the amplitude ($C_{\alpha,i}$) of one of the components approaches unity ($\omega\tau_c \rightarrow 0$).

For a spin $7/2$ nucleus, such as ^{43}Ca , the decay of the longitudinal magnetization may be approximated by a single exponential for correlation times such that $\omega\tau_c \leq 10$, since $C_{11} > 0.96$ for $\omega\tau_c \leq 10$ ⁸. The decay of the transverse magnetization is more complex, and for $\omega\tau_c$ values ≥ 1 multiexponential behaviour is expected (Table 1). The effect of the 300 μs dead time used due to the long "ring down" of our instrument at this frequency is simply to increase the relative amplitude of the dominant component (see Table 1).

For correlation times such that $\omega\tau_c \approx 1.5$, the NMR signal of a spin $7/2$ nucleus displays a virtual Lorentzian line shape. In fact,

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due to the long dead time ($> 300 \mu\text{s}$) of the instrument when low γ nuclei are studied it will be difficult to observe deviations from Lorentzian line shape at even higher $\omega\tau_c$ values.

The apparent longitudinal relaxation rates R_1 in Figure 1 were calculated from the fit of a simple exponential decay to the relaxation parameters obtained numerically and the apparent transverse relaxation rates R_2 from the half width of the actual superposition of four Lorentzian signals. The full lines in Figure 1a show the result of this procedure, using a quadrupole coupling constant χ of 1 MHz, $\omega = 1.078 \cdot 10^8 \text{ s}^{-1}$. Figure 1b shows the ratio R_2/R_1 as a function of $\omega\tau_c$. The apparent relaxation rates above were calculated taking the effect of the $300 \mu\text{s}$ dead time into account, Table 1.

It has recently been shown⁹, by a perturbation treatment, that simple analytical expressions for the relaxation rates exist for "nearly exponential" relaxation. The equations derived, which are identical to those for the average relaxation rates $\langle R_1 \rangle$ and $\langle R_2 \rangle$ derived by McLachlan¹⁰, is given by Equation (3) and (4) for the case of quadrupolar relaxation,

$$\langle R_1 \rangle = \frac{3\pi^2}{10} \chi^2 \frac{2I + 3}{I^2(2I - 1)} \left[\frac{0.2\tau_c}{1 + (\omega\tau_c)^2} + \frac{0.8\tau_c}{1 + (2\omega\tau_c)^2} \right] \quad (3)$$

$$\langle R_2 \rangle = \frac{3\pi^2}{10} \chi^2 \frac{2I + 3}{I^2(2I - 1)} \left[0.3\tau_c + \frac{0.5\tau_c}{1 + (\omega\tau_c)^2} + \frac{0.2\tau_c}{1 + (2\omega\tau_c)^2} \right] \quad (4)$$

Results of calculations based on these equations are given in Figure 1 as dotted lines. The agreement between the analytical expressions and the apparent relaxation rates obtained numerically is good up to $\omega\tau_c \approx 1.0$.

Direct observation of the ^{43}Ca NMR signal from Ca^{2+} ions bound to proteins is possible either under slow exchange conditions (i.e. when the exchange rate, k_{off} , between the protein binding sites and the bulk solution is slow compared to the relaxation rate on the

protein) or when the association constant is sufficiently high to ensure that all of the Ca^{2+} ions are bound to the protein (at low Ca^{2+} concentrations). In practice this restricts us to direct observations of ^{43}Ca resonances from Ca^{2+} binding sites with association constants greater than $\sim 10^6 \text{ M}^{-1}$.

RESULTS

Parvalbumin:

Parvalbumins form a group of relatively small muscle proteins ($M_w = 11.5 \cdot 10^3$ D) found in significant amount in the muscles of aquatic vertebrates.¹¹ The crystal structure of the carp component pI = 4.25 is known from X-ray studies. Parvalbumin contains two high affinity cation binding sites that bind Ca^{2+} and Mg^{2+} with association constants of the order of 10^9 M^{-1} and 10^5 M^{-1} , respectively.¹² In these sites, generally termed the EF and CD sites of PA, the Ca^{2+} ions are octahedrally coordinated by carbonyl and carboxylate oxygen atoms (in the EF site there is one water oxygen coordinated to the Ca^{2+} ion). In addition to the sites described above, ¹¹³Cd NMR measurements show the presence of a "third" cation binding site for carp pI 4.25 (PA) which binds monovalent ions ($K_w \sim 10 \text{ M}^{-1}$) and divalent ions ($K_w \sim 3 \cdot 10^2 \text{ M}^{-1}$). This site is located near one of the strong sites, in fact less than 7 Å away (Swärd et al. to be published). At the present time it is not possible to say if this site is located near the EF or the CD site, since the basis for the assignment of the ¹¹³Cd signals from PA¹³ is still uncertain.

Figure 2 shows the ⁴³Ca NMR signal for different $[\text{Ca}^{2+}]/[\text{PA}]$ ratios at 23°C. The signals seen for $[\text{Ca}^{2+}]/[\text{PA}]$ ratios less than 2 are the ⁴³Ca resonances from Ca^{2+} ions bound to the EF and CD sites of PA. In these spectra, no ⁴³Ca signal is observed from free Ca^{2+} ions since the concentration of these ions is exceedingly low under the experimental conditions. The ⁴³Ca signal is shifted 10 ppm down-field relative to the signal of free Ca^{2+} . The line shape appears to be Lorentzian, with a line-width at half height, $\Delta\nu_{1/2}$ of $670 \pm 30 \text{ Hz}$. At $[\text{Ca}^{2+}]/[\text{PA}]$ ratios greater than 2, an additional narrow ⁴³Ca NMR signal emerges. This signal is 64 Hz broad at room temperature (23°C) while the ⁴³Ca signal in the absence of added protein is 10 Hz. This second ⁴³Ca signal is therefore broadened due to interaction with the EF and CD sites and/or the "third" site.

Figure 3 shows the temperature dependence of the ^{43}Ca spectrum at a $[\text{Ca}^{2+}]/[\text{PA}]$ ratio of 2.4. At increasing temperatures the ^{43}Ca signal from "free" Ca^{2+} is broadened while the line-width of the "protein-bound" signal decreases. The decrease in the line width of the ^{43}Ca signal is partly an effect of the correlation time, τ_c (cf. Equation 4 and Figure 1), which most likely describes the reorientation of the entire protein molecule, and is partly due to the increasing rate of exchange with the free Ca^{2+} ions.

The broadening of the ^{43}Ca signal from the "free" Ca^{2+} ions can readily be explained by chemical exchange between the EF and/or CD sites of PA and the bulk solution. If the exchange rate, k_{off} , is slow compared to the relaxation rate, R_2 , at the binding site, the observed line width of "free" Ca^{2+} increases with increasing exchange rate (i.e. temperature). A rough estimate of the exchange rate of Ca^{2+} at 23°C can be obtained from a band shape analysis of the spectra in Figure 3. The result of the fitting is $k_{\text{off}} = 20 \text{ s}^{-1}$. The question may be raised whether the "third" site contributes significantly to the observed broadening of the "free" Ca^{2+} signal. This type of weak cation site is not likely to bind Ca^{2+} in such a way that slow exchange conditions would apply. The ratio $k_{\text{on}}/k_{\text{off}} = K_w$ gives a value of $k_{\text{off}} > 3 \cdot 10^5 \text{ s}^{-1}$ ($k_{\text{on}} > 10^8 \text{ s}^{-1} \text{ M}^{-1}$, $K_a \approx 3 \cdot 10^2 \text{ M}^{-1}$) which is much greater than the expected value of the relaxation rate of ^{43}Ca at the "third" site. ^{43}Ca ions interacting with this site should then show a "fast exchange" behaviour in variance with the observed behaviour, cf. Figure 3.

Figure 4a shows some partially relaxed ^{43}Ca NMR spectra of the protein-bound Ca^{2+} . Figure 4b shows the intensity of the signals as a function of the delay time, τ ; the data were fitted as a single exponential decay. The result of the fitting procedure is shown as the full line, corresponding to the longitudinal relaxation rate $R_1 = 1.6 \pm 0.1 \cdot 10^3 \text{ s}^{-1}$. The transverse relaxation rate was estimated from the ^{43}Ca signal line-width to be $R_2 = 2.1 \pm 0.1 \cdot 10^3 \text{ s}^{-1}$. All data were measured at 23°C . From this data, using Figure 1b, a correlation

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time of 4.0 ± 1 ns is obtained, a value which is in good agreement with the rotational correlation time calculated from the Debye Stokes-Einstein equation (Table 2). The ^{43}Ca correlation time and the transverse relaxation rate, inserted into the numerical solutions, or into Equation (4), give a value of the quadrupole coupling constant $\chi = 1.3 \pm 0.2$ MHz.

A longer rotational correlation time for PA, 12 ns, has been reported by Nelson et al.¹⁵ from ^{13}C NMR measurements. However, their data were obtained at a higher protein concentration, 15 mM (~20 mass%) which should be compared to 5.6 mM (~8 mass%) used in our ^{43}Ca measurements. The difference in the two correlation times is most likely due to the protein - protein interaction having a non-negligible effect on the rotational diffusion at the higher PA concentration. This argument is supported by ^1H NMR and ^{17}O NMR measurements^{16,17} which demonstrate that at protein concentrations greater than ~ 10 mass%, interactions between the macromolecules become significant.

Troponin C:

Troponin C (TnC), the Ca^{2+} binding component of the troponin complex in vertebrate skeletal muscle, is a low molecular weight protein ($18 \cdot 10^3 \text{D}$). TnC has been reported to possess several classes of cation binding sites: two "high affinity" sites that bind Ca^{2+} and Mg^{2+} with association constants of $2 \cdot 10^7 \text{M}^{-1}$ and $5 \cdot 10^3 \text{M}^{-1}$ respectively, and two weaker "regulatory" binding sites ($K_{\text{Ca}} \approx 3 \cdot 10^5 \text{M}^{-1}$)¹⁸. From sequence analogies¹⁹ these two types of sites are believed to display similarities with the EF and the CD sites of PA. As in the case of PA there is evidence for other weaker cation sites.^{18,20,3} Figure 5a shows some partially relaxed ^{43}Ca NMR spectra of a TnC solution ($[\text{Ca}^{2+}]/[\text{TnC}] = 1.7$) at 23°C . Under the conditions used, only the "high affinity" sites will be populated, and the signal originates from the Ca^{2+} ions bound to these two sites. The signal is shifted 10 ppm downfield relative to the signal of free Ca^{2+} ions and exhibits a Lorentzian line shape with a line width at half height of 750 ± 30 Hz, corresponding to $R_2 = 2.4 \pm 0.1 \cdot 10^3 \text{s}^{-1}$. The data in Figure 5 can be

analysed to obtain the longitudinal relaxation rate. A non-linear fit of the intensities results in $R_1 = 1.0 \pm 0.1 \cdot 10^3 \text{ s}^{-1}$. The resultant ratio $R_2/R_1 = 2.45 \pm 0.45$ corresponds to a correlation time τ_c of $11.0 \pm 2 \text{ ns}$. The value of the correlation time most likely corresponds to the correlation time for the rotational diffusion of the protein molecule (Table 2). Using this value of the correlation time, a quadrupole coupling constant of $1.05 \pm 0.05 \text{ MHz}$ can be estimated from the numerically obtained average relaxation rate.

At higher $[\text{Ca}^{2+}]/[\text{TnC}]$ ratios the ^{43}Ca signal shifts upfield and the line width decreases because of chemical exchange effects from the other sites of TnC^3 .

Calmodulin:

Calmodulin (CaM) is a low molecular weight protein ($M_w = 16.7 \cdot 10^3 \text{ D}$) which acts as a universal regulator of several enzyme systems. The modulation of enzymatic activities takes place after a conformational change induced by the binding of Ca^{2+} .²¹ Like TnC , CaM has 4 regions with an amino acid sequence analogous to those of the EF and CD sites of PA.²² The values of the Ca^{2+} binding constants are still debated,^{21,23} but ^{113}Cd measurements indicate the presence of two high affinity sites comparable to those of TnC .⁴

Figure 6a shows some partially relaxed ^{43}Ca NMR spectra of a CaM solution, $[\text{Ca}^{2+}]/[\text{CaM}] = 1.7$, at 23°C . The observed ^{43}Ca NMR signal is due to the Ca^{2+} ions bound to the "high affinity" sites of CaM. According to published binding constants^{21,23} only the "high affinity" sites will be populated under the conditions used. As is found for PA and TnC the resonance is shifted 10 ppm downfield relative to the signal of free Ca^{2+} and has a line width of $770 \pm 50 \text{ Hz}$ ($R_2 = 2.4 \pm 0.2 \cdot 10^3 \text{ s}^{-1}$). As in the TnC case, the ^{43}Ca resonance shifts towards free Ca^{2+} as the $[\text{Ca}^{2+}]/[\text{CaM}]$ ratio is increased above 2. This effect is attributed to chemical exchange of the bulk Ca^{2+} ions

with the weaker cation sites on CaM (Andersson et al., manuscript in preparation). The data in Figure 6a can be analysed to obtain the longitudinal relaxation rate; a non-linear fit of the intensities results in $R_1 = 1.2 \pm 0.1 \cdot 10^3 \text{ s}^{-1}$. Using the relaxation data and the numerically calculated average relaxation rates, or Equations (3) and (4), we find a correlation time $\tau_C = 8.2 \pm 2 \text{ ns}$ and a quadrupolar coupling constant χ of $1.15 \pm 0.05 \text{ MHz}$. The value of the correlation time is in good agreement with the rotational correlation time, $\tau_C = 8.0 \text{ ns}$, calculated from the Debye-Stokes-Einstein equation, using a Stokes radius of $20.9 \text{ \AA}$,²¹ Table 2.

CONCLUDING DISCUSSION

The chemical shift range of ^{43}Ca is ca 60 ppm, ± 30 ppm relative to free Ca^{2+} ions in aqueous solution²⁴. The chemical shifts of $^{43}\text{Ca}^{2+}$ ions bound to the three proteins all show the same shift value and a similar magnitude of the quadrupole coupling constant (Table 2). This observation supports the arguments, based on sequence homologies, that the Ca^{2+} binding sites in these proteins have the same arrangement of oxygen ligands coordinating the Ca^{2+} ions.

Measurement of R_1 and R_2 for the "protein-bound" ^{43}Ca NMR signals results in correlation times that are in good agreement with the values expected for the rotational correlation time (see Table 2). This finding may be taken to indicate that internal mobility at the Ca^{2+} sites is restricted and considerably slower than the nanosecond time scale.

The use of ^{43}Ca NMR in the study of Ca^{2+} -protein interactions has been shown to be possible using specially designed "high field" instruments in combination with isotopically enriched $^{43}\text{Ca}^{2+}$. At reasonably high protein concentrations "protein-bound" ^{43}Ca resonances can be observed and at relatively short correlation times ($\omega\tau_c \leq 1.5$), information can be obtained about the correlation time and the quadrupole coupling constant from numerically fitted apparent relaxation rates. The analytical equations (Equations (3) and (4)) for the average relaxation rates have been shown to be valid up to $\omega\tau_c \sim 1.0$. For correlation times such that $\omega\tau_c > 2$, a numerical fit of the signal line shape to the relaxation equations is necessary. In this type of calculation the effect of the instrument dead time has to be taken into account.

The present long "ring down" time ($> 300 \mu\text{s}$) of NMR spectrometers operating at this frequency (17.16 MHz) results in a loss of $\sim 50\%$ of the signal intensity ($\chi = 1 \text{ MHz}$) due to relaxation during the dead time. An additional disadvantage is that a long dead time makes it difficult to observe a non-Lorentzian line shape. This indicates a need for probe constructions that lead to shorter "ring down" times.

ACKNOWLEDGEMENTS

We thank Bertil Halle for valuable discussion on relaxation theory. One of us, T.A., gratefully acknowledges a grant from Stiftelsen Bengt Lundqvists Minne.

Note added in proof:

It has recently been pointed out that second order effects may influence the NMR signal line-shape under non-extreme narrowing conditions²⁵. Clearly these dynamic frequency shifts should be included in the analysis of the ^{43}Ca NMR spectra described in this work. It can however be shown (H. Wennerström, private communication) that neglect of these effects only produces small errors for parameters applicable to this work: $\omega\tau_c \ll 1.5$, $\omega \approx 1 \cdot 10^8 \text{ s}^{-1}$ and $\chi = 1 \text{ MHz}$.

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Table 1. The relaxation data for a spin 7/2 nucleus at $\omega\tau_C = 1.5$, $\omega = 1.078 \cdot 10^8 \text{ s}^{-1}$ and $\chi = 1 \text{ MHz}$

Relative amplitude coefficient		Relaxation rate ^a (s^{-1})	Apparent relaxation rate ^b	
$t_d = 0$	$t_d = 300 \text{ } \mu\text{s}$		$t_d = 0$ (s^{-1})	$t_d = 300 \text{ } \mu\text{s}$
$C_{2,1} = 0.832$	$C_{2,1} = 0.912$	$R_{2,1} = 2.12 \cdot 10^3$	$R_2 = 2.22 \cdot 10^3$	$R_2 = 2.17 \cdot 10^3$
$C_{2,2} = 0.083$	$C_{2,2} = 0.068$	$R_{2,2} = 3.09 \cdot 10^3$		
$C_{2,3} = 0.079$	$C_{2,3} = 0.019$	$R_{2,3} = 7.13 \cdot 10^3$		
$C_{2,4} = 0.006$	$C_{2,4} = 0.0002$	$R_{2,4} = 12.93 \cdot 10^3$		

^a The relative amplitudes immediately after the pulse ($t_d = 0$) and after the instrument dead time ($t_d = 300 \text{ } \mu\text{s}$) described in ref. 8.

^b The apparent relaxation rates obtained from the line width at half height of the actual superposition of 4 lorentzian lines at $t_d = 0$ and $t_d = 300 \text{ } \mu\text{s}$.

Table 2. Some physical constants and ^{43}Ca NMR data for PA, CaM and TnC

Protein	M_w (10^3D)	Hydrodynamic radius		τ_{rot}^a (ns)	R_2/R_1	τ_c (ns)	δ (ppm)	χ (MHz)
		R_H (nm)	R_H (nm)					
Parvalbumin	11.5	1.5	1.5	2.9	1.35 ± 0.15	4.0 ± 1	$+10 \pm 1$	1.3 ± 0.2
Calmodulin	16.8	2.1	2.1	8.0	2.05 ± 0.35	8.2 ± 2	$+10 \pm 1$	1.15 ± 0.05
Troponin C	17.8	2.2	2.2	9.3	2.45 ± 0.45	11.0 ± 2	$+10 \pm 1$	1.05 ± 0.05

^a For a sphere of radius R_H , obeying the Debye-Stokes-Einstein Relation $\tau_{\text{rot}} = 4\pi\eta R_H^3 / (3 kT)$, where

$$T = 296 \text{ K and } \eta = 8.50 \cdot 10^{-4} \text{ kg (ms)}^{-1}.$$

FIGURE LEGENDS

Figure_1 a) The full lines represent the apparent relaxation rates, calculated from a fit of a simple exponential decay (for R_1) to the numerically obtained relaxation parameters and from the half width of the actual superposition of Lorentzian lines (for R_2). In the calculations above the effect of the finite dead time (300 μ s) is taken into account. The dotted lines represent the analytical expressions for the average relaxation rates, Equation (3) and (4). All data was calculated using $\omega = 1.078 \cdot 10^8 \text{ s}^{-1}$ and $\chi = 1 \text{ MHz}$.

b) The ratio R_2 / R_1 as a function of $\omega\tau_c$. The data were calculated as described above.

Figure_2 The ^{43}Ca NMR spectrum of a 5.6 mM PA solution, at 23°C and pH = 7.3, at different $[\text{Ca}^{2+}]/[\text{PA}]$ ratios. The spectra were recorded using a 70 μ s (90°) pulse, a data acquisition time of 20 ms and an instrument dead time of 300 μ s. Each spectrum required ca. $2 \cdot 10^6$ transients corresponding to ~ 12 hours of accumulation.

Figure_3 The temperature dependence of the ^{43}Ca NMR spectrum of a 5.6 mM PA solution, pH 7.3, at a $[\text{Ca}^{2+}]/[\text{PA}]$ ratio of 2.4. The data were recorded using a pulse repetition rate of 40 ms, a dead time of 300 μ s and a 70 μ s pulse (90°).

Figure_4 a) The ^{43}Ca NMR spectrum of a 5.6 mM PA solution, 23°C and pH 7.3, at a $[\text{Ca}^{2+}]/[\text{PA}]$ ratio of 1.9 after different delay times in the inversion recovery sequence.

b) The intensities of the signals as a function of the delay time. The solid curve represents the result of fitting the data to the Equation:

$$Y = A(1 - \text{Bexp}(-t/T_1))$$
, resulting in $A = 11.6$, $B = 1.9$ and $T_1 = 0.64 \text{ ms}$

Figure_5 a) Some partially relaxed ^{43}Ca NMR spectra of a 1.0 mM TnC solution, pH 7.2, containing 1.75 mM Ca^{2+} . The spectra were taken at 23°C . Each spectrum was recorded using a pulse interval of 10 ms, a 90° pulse of 50 μs and a "ring down" time of 300 μs . For each spectrum a total of $2 \cdot 10^6$ transients were recorded during ca 6 hours of accumulation.

b) The intensities of the signals above as a function of the delay time τ . The solid line represents a fit of the equation given in the caption to Figure 4 to the data, yielding $A = 19.0$, $B = 1.6$ and $T_1 = 0.95$ ms.

Figure_6 a) The ^{43}Ca NMR spectrum of a 2.8 mM CaM solution, pH 7.3 containing 1.7 moles of Ca^{2+} per mole of protein, at different delay times in the inversion recovery sequence. Each spectrum was recorded using a pulse interval of 20 ms, a pulse width of 100 μs (90°) and an instrumental dead time of 300 μs . For each spectrum $2 \cdot 10^6$ transients were recorded during ca 12 hours of accumulation.

b) The intensity of the signals above as a function of the delay time. The solid line represents the result of fitting the data to the equation given in the caption to Figure 4, yielding $A = 9.8$, $B = 1.6$ and $T_1 = 0.86$ ms.

Figure 1.

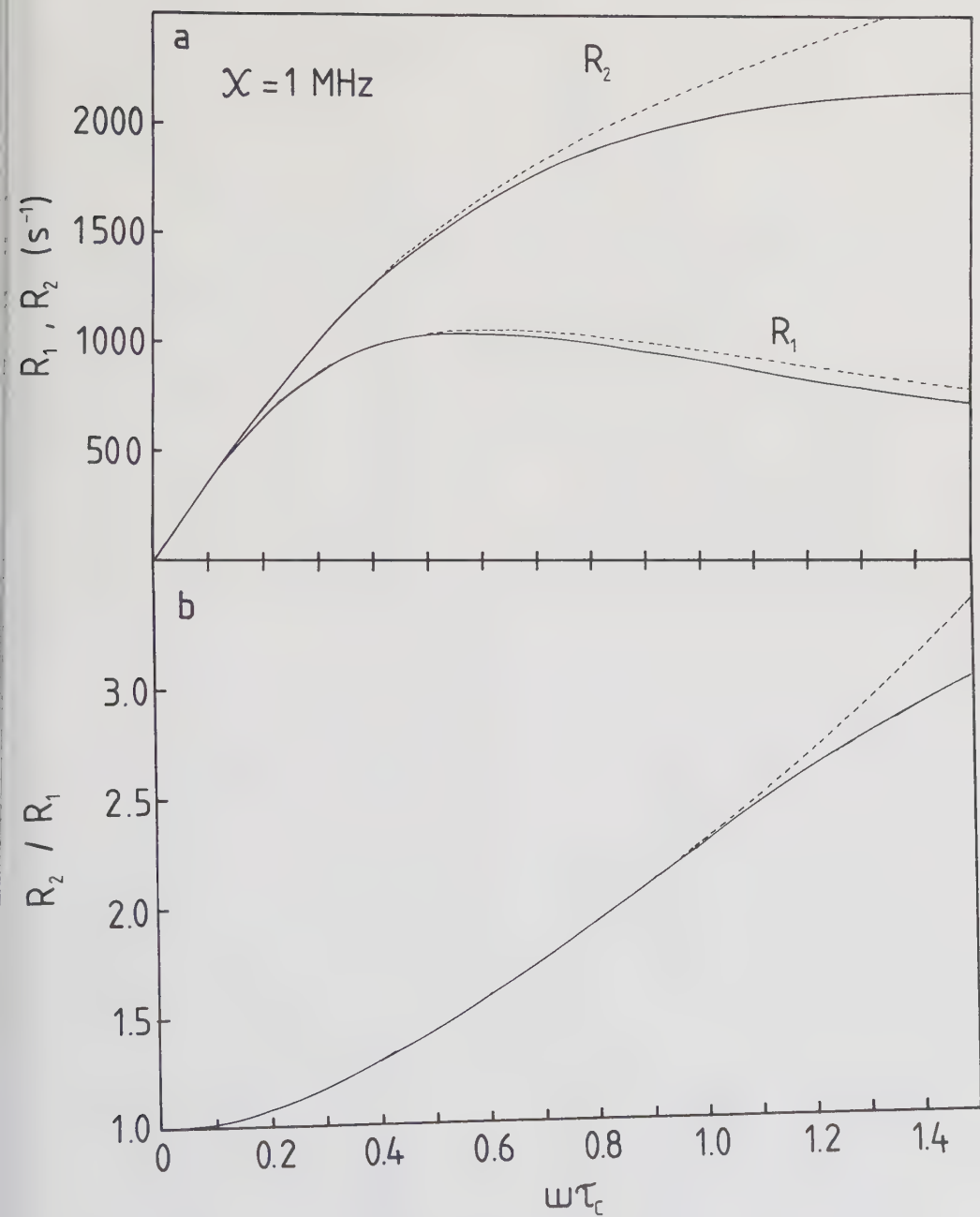


Figure 2.

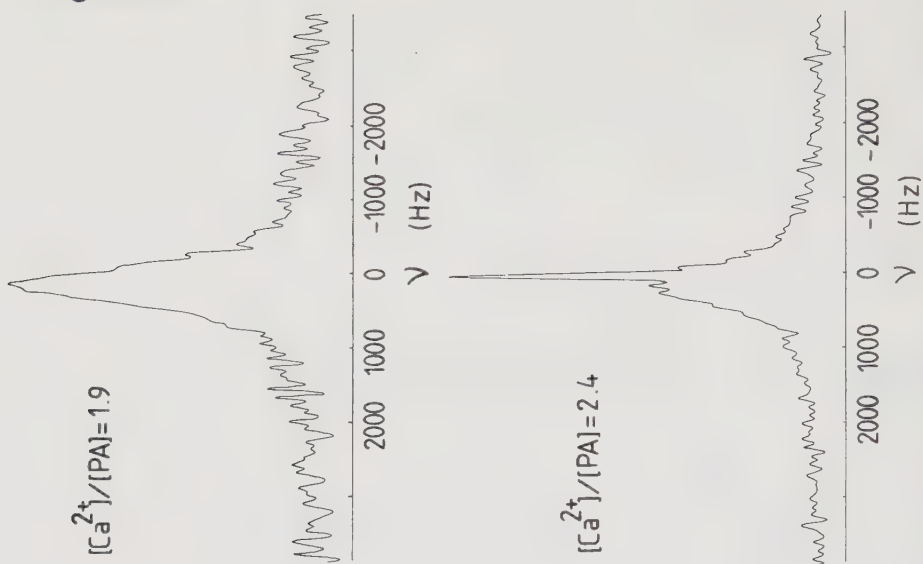
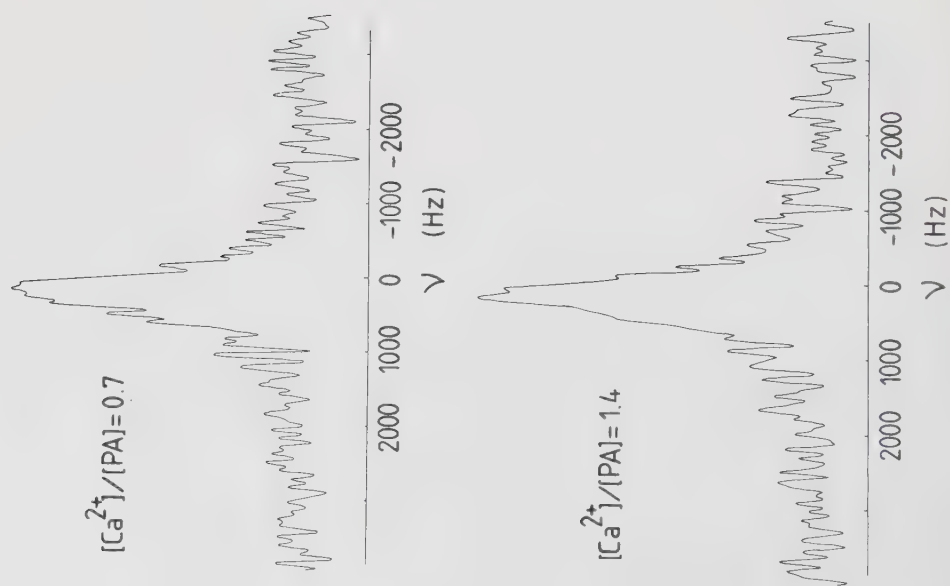


Figure 3.

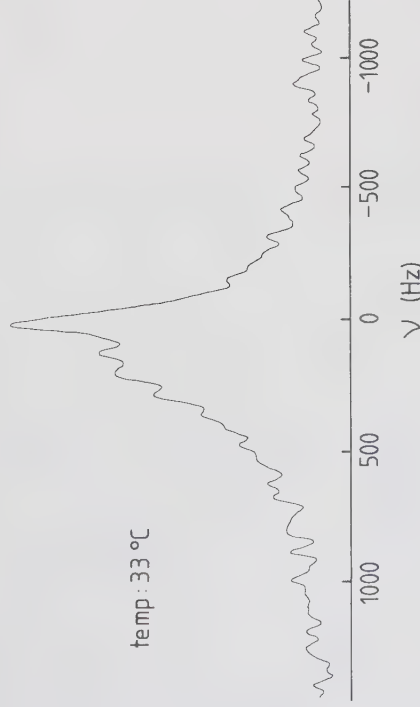
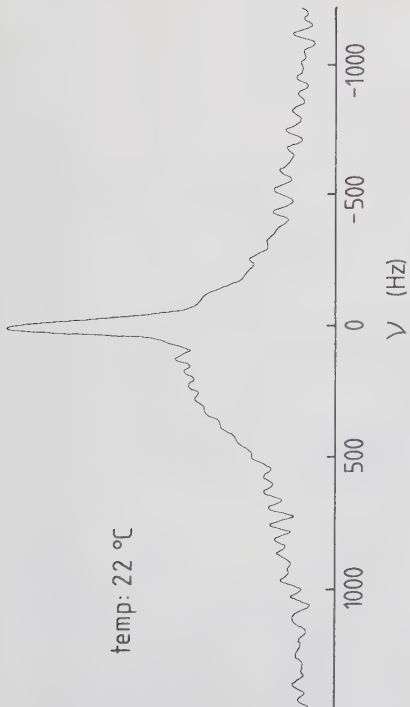
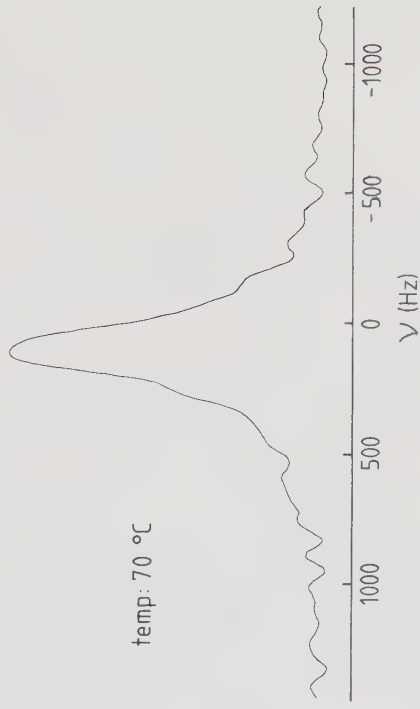
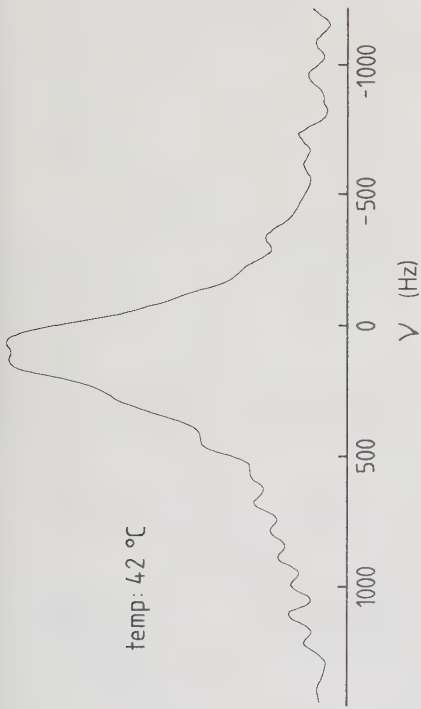
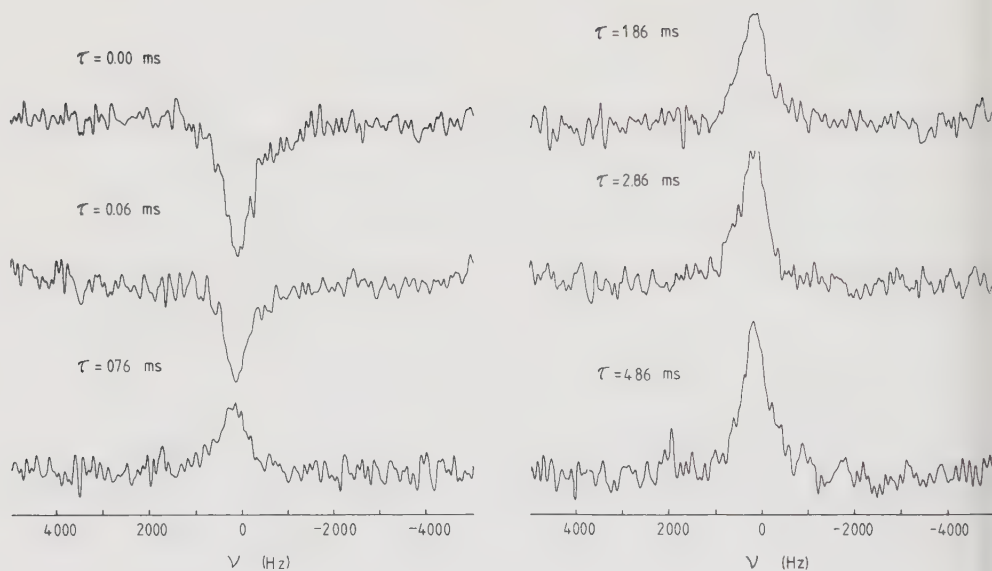


Figure 4.

a

PA



b

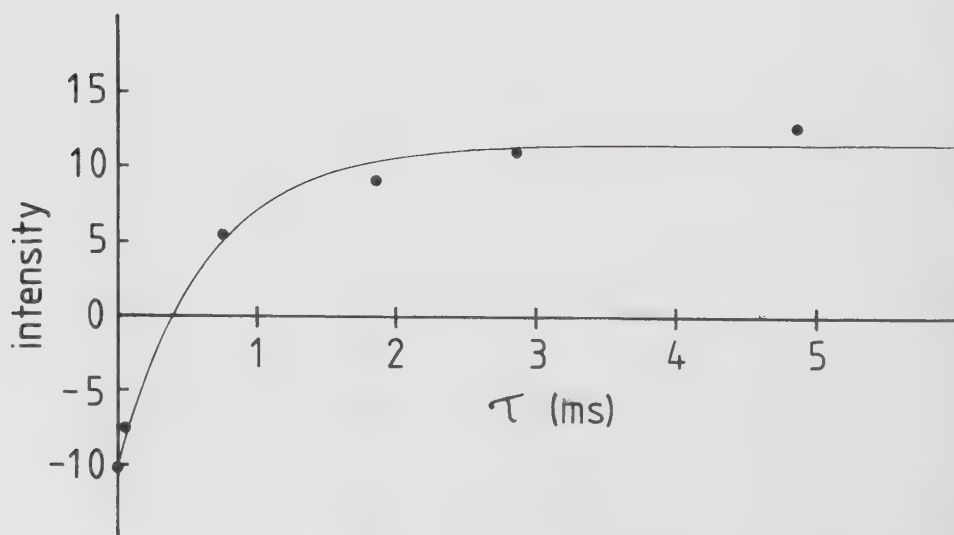


Figure 5.

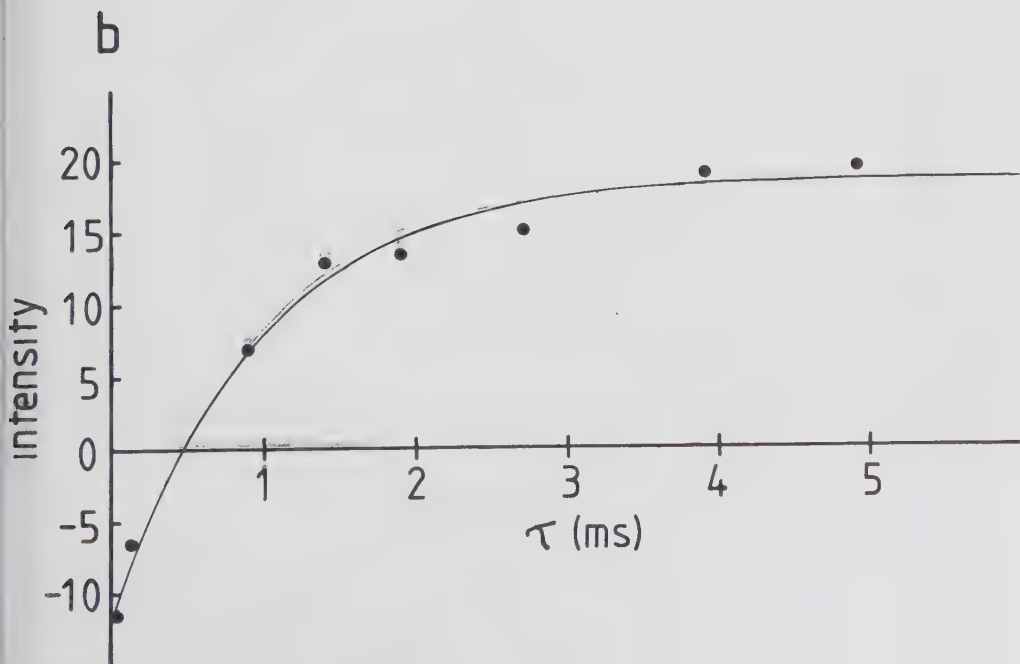
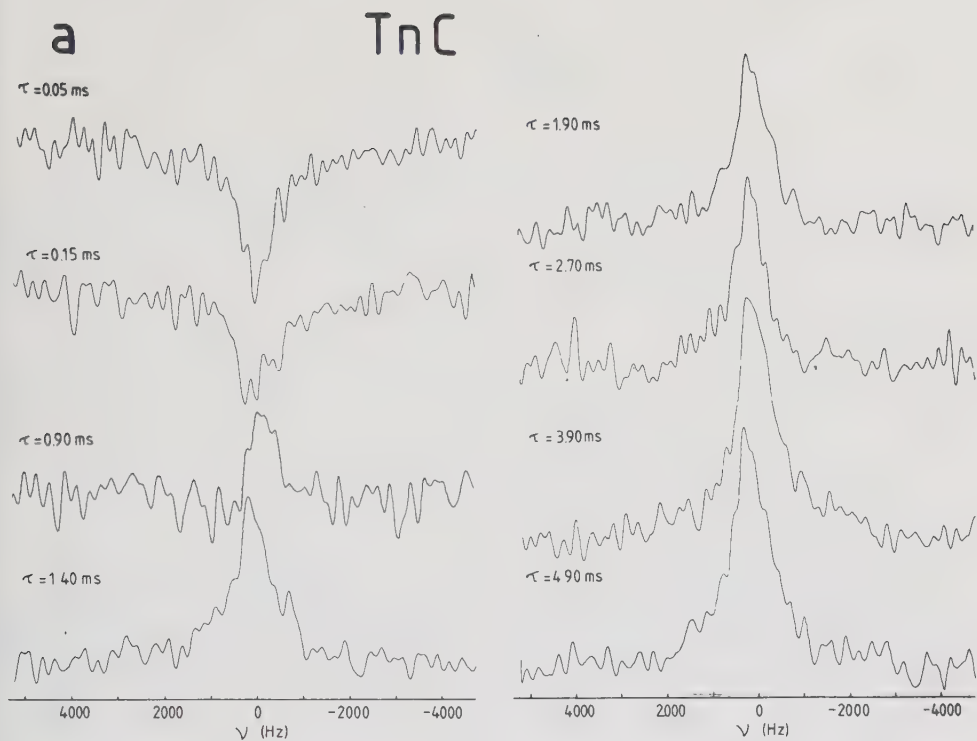
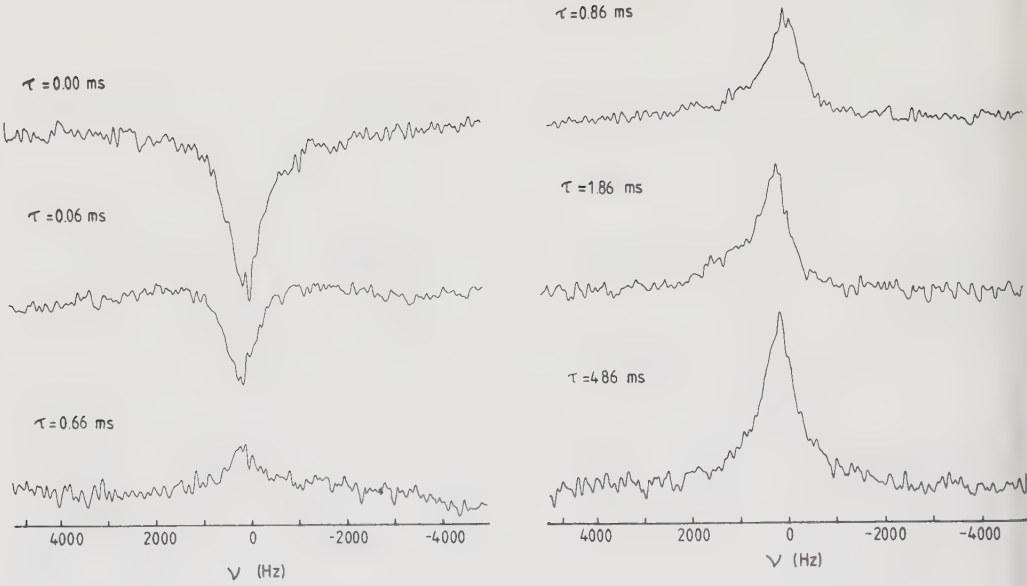
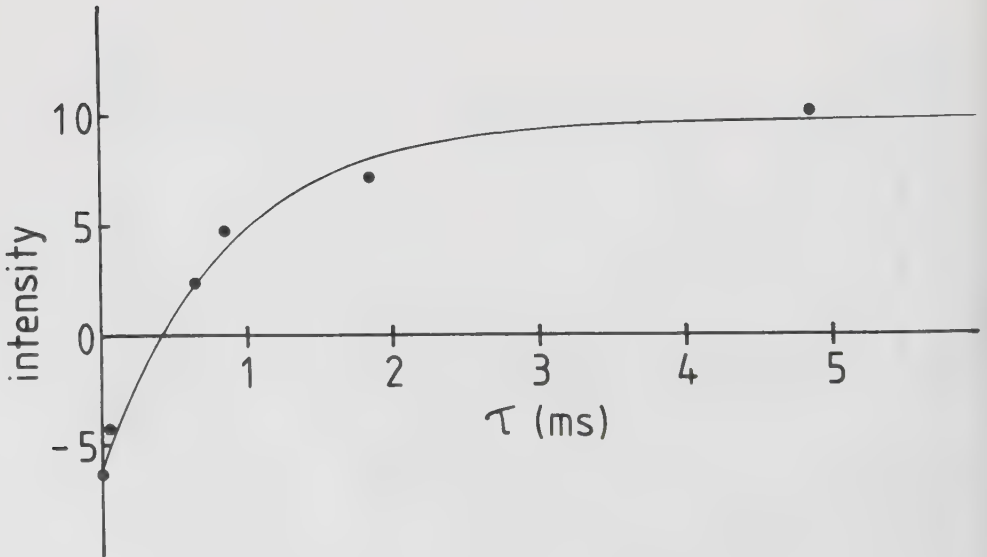


Figure 6

a CaM



b



ARTICLE VII

Characterization of Cation Binding Sites on Panulirus interruptus
Hemocyanin by ^{43}Ca and ^{23}Na NMR.

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Running title: Cation Binding to Hemocyanin

NOTES:

1. Abbreviations used:

NMR, nuclear magnetic resonance

HC, Panulirus interruptus hemocyanin

FOOTNOTES:

- * It has recently been pointed out that second order effects may influence the the NMR signal line shapes under non-extreme narrowing condotions. Clearly these dynamic frequency shifts should be included in the analysis of NMR spectra. It can however be shown that neglection of these effects only will produce minor errors in the obtained relaxation rates $R_{2,1}$ and $R_{2,2}$.

INTRODUCTION

Hemocyanins are the copper-containing respiratory proteins of arthropods and molluscs. In the proteins from both phyla the oxygen binding site comprises two copper atoms but the size of the polypeptide chains differs. Molluscan hemocyanins are made up by large polypeptide chains ($M_r \sim 2-3 \cdot 10^5$) each carrying 7-8 oxygen binding sites while arthropod hemocyanins are made up of smaller subunits ($M_r \sim 7-8 \cdot 10^4$) each carrying one oxygen binding site [1].

The effects of calcium, magnesium, sodium and hydrogen ions on the structure and biological function of hemocyanins have been known for many years [1]. Only recently however has attention been directed to the analysis of the specific and reciprocal effects arising from the interplay of all these allosteric ligands in the case of the relatively small hemocyanin from the spiny lobster Panulirus interruptus (a hexamer of $M_r \sim 4.5 \cdot 10^5$). This task demands a complete characterization of the different sets of binding sites for each ligand. The present paper focuses on the binding of Ca^{2+} to Panulirus hemocyanin (HC). This was measured directly by means of ^{43}Ca NMR and indirectly by studying the effect of Ca^{2+} on the ^{23}Na line width. The combined use of ^{43}Ca and ^{23}Na NMR allowed two different classes of Ca^{2+} binding sites differing approximately 10-fold in affinity, to be distinguished. Information on the dynamics of the interaction of the protein binding sites has been obtained.

MATERIALS AND METHODS

Hemocyanin was isolated and fractionated as described by Kuiper et al. [2] and van der Berg [3]. For this study, reassociated fraction II, which showed the same physico-chemical and functional properties as the unfractionated material was used. The lyophilized protein was

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dissolved in a 0.02 M Tris buffer at pH 7.2 containing 0.08 M NaCl and 5 mM EDTA and dialysed against one change of the buffer without EDTA. The protein was then dialysed against several changes of NaCl solutions at the desired concentration (EDTA is known to bind strongly to proteins [4] but no residual EDTA was detected after this procedure using ^{113}Ca NMR (Ca^{2+} is known to bind very strongly to EDTA and produces a signal with a characteristic chemical shift). The concentration of the HC solution was determined spectrophotometrically and is expressed in terms of moles of subunit ($M_s = 7.5 \cdot 10^{-4}$) [1]. The pH of the HC solution was changed by adding small amounts of 1 M NaOH or 1 M HCl. All experiments were performed with HC in its native aggregated state ($M_n \approx 4.5 \cdot 10^5$); this was checked in sedimentation velocity experiments carried out at 40000 rev/min in a Spinco Mod E ultracentrifuge.

An aqueous 0.086 M CaCl_2 solution was prepared by dissolving CaCl_2 (60 % isotopically enriched in ^{43}Ca , Oak Ridge Nat. Lab., Oak Ridge) in 1 M HCl. The solution was neutralised to a final pH of 7.0 by adding 1 M NaOH.

^{23}Na and ^{43}Ca NMR spectra were recorded at 67.4 and 17.0 MHz respectively on a laboratory-made Fourier transform spectrometer equipped with an Oxford Instruments 6 Tesla wide bore superconducting magnet (room temperature bore = 89 mm). In order to increase the signal-to-noise ratio for the low-sensitivity ^{43}Ca isotope a probe with horizontal sample orientation was used resulting in an increase in sensitivity by a factor of 2.5 (corresponding to a gain in time by a factor of 6) compared to the conventional vertical sample orientation [5]. All measurements were made at 23°C unless otherwise stated.

The observed non-Lorentzian ^{23}Na absorption spectra were computer-analysed in order to obtain the individual relaxation rates of the two components in the decay of the transverse magnetization [6,7].

The apparent ^{43}Ca NMR relaxation rate was obtained from the line width of the signal at half height ($\Delta\nu_{1/2}$); this was related to the relaxation rate by $R_2 = \pi\Delta\nu_{1/2}$. No deviation from Lorentzian line shape was observed. The apparent ^{43}Ca longitudinal relaxation rate was determined from a non-linear least squares fit of the ^{43}Ca signal intensities in the inversion recovery experiment [8].

THEORY

For a nucleus with a spin quantum number (I) greater than $\frac{1}{2}$, such as ^{43}Ca and ^{23}Na , the distribution of charges over the nucleus deviates from spherical symmetry, a deviation which can be described in terms of a nuclear electric quadrupole moment (eQ). If the electronic distribution around the nucleus has less than cubic symmetry, an electric field gradient at the nucleus will result which will interact with the nuclear quadrupole moment. Fluctuations in the magnitude and/or direction of the field gradient generally provide an efficient mechanism for nuclear magnetic relaxation, a mechanism so efficient that with a few notable exceptions it is the only relaxation mechanism that needs to be considered [9,10].

In the case of a free solvated quadrupolar ion (like $^{23}\text{Na}^+$ and $^{43}\text{Ca}^{2+}$) in aqueous solution, the quadrupole relaxation is relatively inefficient, resulting in comparatively narrow NMR signals (usually < 10 Hz at room temp.). When the ion is bound to a macromolecule binding site however the NMR signals are usually very broad ($> 10^4 - 10^5$ Hz) making a direct observation of the "protein-bound" NMR signals extremely difficult [10]. At the time of writing such observations have been made only for $^{43}\text{Ca}^{2+}$ bound to three proteins, parvalbumin, troponin C and calmodulin [11] under favourable conditions.

Studies of the binding of quadrupolar ions are however possible if there is a reasonably fast chemical exchange of ions between bound and free states [5].

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The magnetic relaxation of an $I > 1$ nucleus is, in general, not a simple exponential decay. For nuclei with half integer spins (^{23}Na $I = 3/2$ and ^{43}Ca $I = 7/2$) the magnetization decays as a weighted sum of $I + \frac{1}{2}$ exponentials [13,14].

^{23}Na NMR. When non-"extreme narrowing" conditions apply (i.e. $\omega\tau_c > 1$, where τ_c is the correlation time describing the fluctuations in the electric field gradients at the nucleus and ω is the resonance frequency in radians/s) the decay of the transverse magnetization (M_2) decays as a weighted sum of two exponentials [12].

$$M_2(t) = M_2(0) [0.6 \exp(-tR_{2,1}) + 0.4 \exp(-tR_{2,2})] \quad (1)$$

In a two-site system with one site holding the free solvated ions and the other the protein binding site, the intrinsic relaxation rates, $R_{2,i}$ when fast exchange conditions apply, are given by the equation [13]

$$R_{2,1} = (1 - p_B)R_2^0 + p_B \left[\frac{\pi^2}{5} \chi^2 \left(\tau_c + \frac{\tau_c}{1 + (\omega\tau_c)^2} \right) \right] \quad (2)$$

$$R_{2,2} = (1 - p_B)R_2^0 + p_B \left[\frac{\pi^2}{5} \chi^2 \left(\frac{\tau_c}{1 + (\omega\tau_c)^2} + \frac{\tau_c}{1 + (2\omega\tau_c)^2} \right) \right] \quad (3)$$

where R_2^0 is the relaxation rate of ^{23}Na in the absence of protein, p_B is the fraction of Na^+ ions bound to the protein and χ is the quadrupole coupling constant (given in Hz). The expressions within the square brackets refer to the relaxation rates of the ^{23}Na components at the protein sites and are denoted $R_{2,1}^B$ and $R_{2,2}^B$. The subscript "1" denotes the "fast-decaying" component of the transverse magnetization. The excess relaxation rate is defined by:

$$R_{2,i}^{\text{Ex}} = R_{2,i} - R_{2,0} \quad (i=1,2 \text{ and } p_B \ll 1).$$

The high resolution NMR spectrum of a ^{23}Na nucleus at $\omega\tau_c > 1$ will be a superposition of two Lorentzian signals having the same

frequency*, but with line widths corresponding to $R_{2,1}$ (60 % intensity) and $R_{2,2}$ (40 % intensity).

^{43}Ca NMR. The decay of the longitudinal (M_1) and transverse (M_2) magnetization for a spin 7/2 nucleus such as ^{43}Ca is a weighted sum of 4 exponentials [14].

$$M_1(t) = M(\infty) \left[1 + k \sum_{i=1}^4 C_{1,i} \exp(-t R_{1,i}) \right] \quad (4)$$

$$M_2(t) = M(0) \left[\sum_{i=1}^4 C_{2,i} \exp(-t R_{2,i}) \right] \quad (5)$$

where $\sum_{i=1}^4 C_{\alpha,i} = 1$ and $k = 2$ in the inversion recovery experiment.

It is not possible to obtain analytical solutions of the relaxation equations, but they may be solved numerically for each set of $\omega\tau_c$ values [14]. A single exponential decay is approached as the relaxation rates of the components become increasingly alike and/or as the amplitude of one of the components approaches unity. The longitudinal and transverse magnetization behave differently. The decay of the longitudinal magnetization is expected to be virtually exponential for $\omega\tau_c$ values ≤ 10 [14]. Pronounced deviation from a simple exponential decay of the transverse magnetization is however to be expected for $\omega\tau_c$ values greater than 2 [14]. When the relaxation is nearly exponential an apparent relaxation rate can be defined from a fit of a single exponential decay or of a Lorentzian signal to the relaxation data numerically obtained [15,11]. Figure 1 shows the apparent transverse and longitudinal relaxation rates and the ratio R_2/R_1 as a function of $\omega\tau_c$.

It has recently been shown [15] by a perturbation treatment that simple analytical expressions for the apparent relaxation rates exist for "nearly exponential" relaxation. The result, which is

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identical to the average relaxation rates derived by McLachlan [16], is given in Equation (6) and (7) for the case of quadrupole relaxation, and $I = 7/2$.

$$\langle R_1 \rangle = \frac{2\pi^2}{49} \chi^2 \left[\frac{0.2 \tau_c}{1 + (\omega\tau_c)^2} + \frac{0.8 \tau_c}{1 + (2\omega\tau_c)^2} \right] \quad (6)$$

$$\langle R_2 \rangle = \frac{2\pi^2}{49} \chi^2 \left[0.3 \tau_c + \frac{0.5 \tau_c}{1 + (\omega\tau_c)^2} + \frac{0.2 \tau_c}{1 + (2\omega\tau_c)^2} \right] \quad (7)$$

The equations are given as the dotted lines in Figure 1. Their limits of applicability have been tested for a two-site system with fast chemical exchange [15].

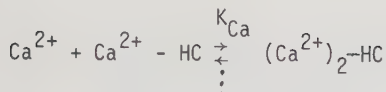
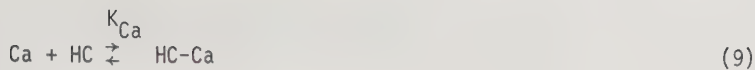
If the chemical exchange rate, k_{off} , is much faster than the relaxation rate at the protein sites, (R_α^B), the relaxation rate of the observed ^{43}Ca NMR signal (R_α^{OBS}) will be a weighted average according to:

$$R_\alpha^{\text{OBS}} = (1 - p_B) R_\alpha^0 + p_B R_\alpha^B \quad (\alpha = 1, 2) \quad (8)$$

where R_α^0 is the ^{43}Ca relaxation rate of "free" $^{43}\text{Ca}^{2+}$ ions and p_B is the fraction of bound ions. By measuring both the transverse and longitudinal apparent relaxation rates the correlation time is obtained from Figure 1b or Equation (6) and (7).

RESULTS

^{43}Ca NMR. Figure 2 shows the apparent ^{43}Ca NMR relaxation rate of a 1.0 mM CaCl_2 solution at neutral pH as a function of the concentration of added HC. The data shows typical saturation behaviour (i.e. $p_B \rightarrow 1$ in Equation (8)) although ^{43}Ca spectra at higher $[\text{HC}]/[\text{Ca}^{2+}]$ ratios could not be obtained due to experimental limitations in the HC concentration. The data were fitted to the so called isodesmic model where HC possesses n equivalent and independent sites per subunit.



In this model the fraction of ions bound to the protein is obtained from the equation:

$$p_B = \left[\frac{1}{2} (C_{\text{Ca}} + nC_{\text{HC}} + 1/K_{\text{Ca}}) - \sqrt{\frac{1}{4} (C_{\text{Ca}} + nC_{\text{HC}} + 1/K_{\text{Ca}})^2 - nC_{\text{HC}} C_{\text{Ca}}} \right] / C_{\text{Ca}} \quad (10)$$

where C_{Ca} is the total Ca^{2+} concentration and C_{HC} is the total HC concentration. Equation (12), inserted into Equation (8) was fitted to the ^{43}Ca relaxation data in Figure 2. The result of the fitting procedure indicates the presence of 4 to 16 sites per subunit having association constants in the range of $1-5 \cdot 10^3 \text{ M}^{-1}$. The relaxation rate of the bound $^{43}\text{Ca}^{2+}$ ions was estimated to be $R_2^B = 8 \pm 2 \cdot 10^2 \text{ s}^{-1}$.

No significant differences in the ^{43}Ca relaxation parameters were observed when going from deoxy HC to oxy HC, in contrast to the reported decrease in the ^{23}Na line width (7).

Figure 3 shows the Ca^{2+} concentration dependence of the apparent ^{43}Ca transverse relaxation rate for a 0.33 mM HC solution at neutral pH. The decrease in the relaxation rate occurring with an increase in the total Ca^{2+} concentration, C_{Ca} can be accounted for in terms of a reduction in the fraction of ions bound to HC (p_B in Equation (8) and (10)). The model of Equations (8) and (10) was fitted to the experimental data. The results indicate the presence of 3 - 4 sites per subunit having an association constant K_{Ca} of ca $2 \cdot 10^3 \text{ M}^{-1}$. The relaxation rate at the protein sites is estimated to be $R_2^B = 7 \pm 2 \cdot 10^2 \text{ s}^{-1}$.

Figure 4 shows the temperature dependence of the apparent ^{43}Ca transverse relaxation rates for a 1.0 mM CaCl_2 solution containing HC at neutral and at alkaline pH values. The plot of $\ln R_{2,e}$ versus T^{-1} is linear with a positive slope, indicating that fast exchange conditions apply ($k_{\text{off}} \gg R_2^B$) [9] and that the use of equation (8) is justified. A lower limit for the exchange rate, $k_{\text{off}} > 10^3 \text{ s}^{-1}$, is obtained from

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this experiment and from the values of R_2^B given above.

The pH dependence of the apparent ^{43}Ca transverse relaxation rate for a 1.0 mM CaCl_2 solution, containing 0.11 mM HC is shown in Fig. 5. Measurements could not be performed near the isoelectric point (between pH 4.5 and 6.0) due to the low solubility of the protein. No significant dissociation into subunits occurs up to pH 10 at the protein and Ca^{2+} concentrations used, according to sedimentation velocity experiments.

Figure 6 shows the apparent ^{43}Ca transverse relaxation rate as a function of added Mg^{2+} for a 0.8 mM CaCl_2 solution, pH 6.7, containing 0.43 mM HC. The decrease in the relaxation rate when Mg^{2+} is added is most likely due to a direct $\text{Mg}^{2+} - \text{Ca}^{2+}$ competition for common sites. An analysis of the data along those lines indicates that Ca^{2+} and Mg^{2+} have similar affinities for such sites. Assuming $K_{\text{Ca}} = 1 \cdot 10^3 \text{ M}^{-1}$ the corresponding value for Mg^{2+} is: $K_{\text{Mg}} = 8 \cdot 10^2 \text{ M}^{-1}$.

The apparent transverse and longitudinal relaxation rates were determined for a 3.7 mM CaCl_2 solution, as neutral pH, containing 0.25 mM HC. This data allows calculation of the correlation time τ_c from Figure 1b or Equations (6) and (7) provided that fast exchange conditions apply. The observed ratio $R_{2,\text{ex}}/R_{1,\text{ex}} = 3.0 \pm 0.2$ yields a correlation time $\tau_c = 14 \pm 2 \text{ ns}$. (The same value is obtained for deoxy HC).

By using the estimated value of R_2^B and τ_c , a value of the quadrupole coupling constant can be calculated from the numerically obtained relaxation parameters or Equation (7). The resulting value is $\chi = 0.6 \pm 0.1 \text{ MHz}$.

^{23}Na NMR. Recent experiments [7] showed that when HC is added to a NaCl solution, the ^{23}Na NMR signal is considerably broadened. The line shape is markedly non-lorentzian and the relaxation rates for the fast ($R_{2,1}$) and slow ($R_{2,s}$) decaying components are easily obtained from a band shape analysis of the NMR spectrum [6,7]. Fast

exchange conditions were found to apply [7].

The effect of Ca^{2+} and Mg^{2+} additions on the ^{23}Na line shape has been compared. Figures 7a and 7b show the excess transverse relaxation rates of the fast decaying component as a function of added Ca^{2+} or Mg^{2+} for a 12.0 mM NaCl solution, at neutral pH, containing 0.14 mM HC. The simplest interpretation of the observed decrease in the relaxation rate is that divalent cations compete with Na^+ for the same sites - resulting in a reduction in the fraction of Na^+ ions bound to HC (i.e. p_B in Eq. (2) and (3)). The data may be analysed in terms of the isodesmic model, in which HC is assumed to possess n equal independent cation binding sites per subunit and for which Na^+ and Ca^{2+} will compete.

In the case of Ca^{2+} a computer fit of the model to the relaxation data, assuming $K_{\text{Na}} = 1 \cdot 10^2 \text{ M}^{-1}$ [7], results in $n = 0.7 \pm 0.3$

$$K_{\text{Ca}} = 3 \pm 1 \cdot 10^4 \text{ M}^{-1} \text{ and } R_{2,1}^B = 10^5 \text{ s}^{-1}.$$

In the case of Mg^{2+} , using $K_{\text{Na}} = 1 \cdot 10^2 \text{ M}^{-1}$ and $n = 0.7$, an association constant $K_{\text{Mg}} = 1 \cdot 10^4 \text{ M}^{-1}$ was obtained. A correlation time of $16 \pm 4 \text{ ns}$ can be estimated from the value of $R_{2,1}$ and $R_{2,2}$ according to Equations (2) and (3). The improved sensitivity of the NMR spectrometer used in the present experiments allowed the use of a lower Na^+ concentration and of lower Ca^{2+} to protein ratios than in [7]. This enables us to obtain a more accurate value for the number of Ca^{2+} binding sites.

DISCUSSION

The picture which emerges from the present study is that the combination of results from ^{23}Na and ^{43}Ca NMR allows different Ca^{2+} binding sites on the Panulirus hemocyanin molecule to be distinguished. Thus ^{23}Na NMR shows the presence of ~ 1 strong Ca^{2+} binding site/subunit, while ^{43}Ca NMR indicates the presence of 3-17 sites of lower affinity

(Table I). In the concentration range accessible to ^{43}Ca NMR ($> 1\text{mM}$) the contribution to the observed relaxation rates of Ca^{2+} binding to the "strong sites" is negligible and the "weak sites" dominate the experimental situation. The opposite is true in ^{23}Na NMR where Na^+ ions bound to the "strong sites" have a much greater effect on the ^{23}Na relaxation rate than have Na^+ ions bound to the "weak sites". This is clearly brought out by the observation that most of the excess ^{23}Na relaxation rate vanishes when about 1 Ca^{2+} /subunit is added to Panulirus hemocyanin. The Ca^{2+} binding constants obtained in the present study are in good agreement with those previously reported [17] that have been obtained by means of a Ca^{2+} selective electrode. The latter technique gives an overall binding constant equivalent to $K_{\text{Ca}} \approx 2 \cdot 10^4 \text{ M}^{-1}$ [17].

A unique type of information which can be obtained from ^{43}Ca NMR concerns the dynamics at the Ca^{2+} binding sites. The correlation time calculated for ^{43}Ca ions bound to the "weak sites", $\tau_c = 14 \pm 2 \text{ ns}$, is considerably shorter than the expected value for the rotational diffusion of the entire protein molecule ($\tau_c^{\text{rot}} = \sim 250 \text{ ns}$, calculated from the Debye-Stokes-Einstein equation [18] using a hydrodynamic radius of 6.5 nm [19]). This result indicates a considerable mobility at the calcium binding sites. A similar value of the correlation time $\tau_c = 16 \pm 4 \text{ ns}$ is obtained for ^{23}Na .

Study of the pH dependence of the apparent ^{43}Ca relaxation rate provides information on the nature of the binding sites. In general, changes in the observed relaxation rate may result from a change in the fraction of ions bound (p_B in eq. (8)) or in the relaxation rate at the protein binding site (R_2^B in eq. (8)). The changes in the apparent ^{43}Ca relaxation rate below pH 6.5 are most likely due to the titration of groups responsible for the binding of Ca^{2+} . In spite of the limitations in the accessible pH range, the data indicate an approximate pK value of 5.3 ± 0.3 for those ligands. Similar pK values have been measured in a variety of Ca^{2+} binding proteins where carboxylate side chains are known to be involved in Ca^{2+}

binding [20, 21]. The increase in the ^{43}Ca relaxation rate observed between pH 7 and 10 can be attributed to an increased number of Ca^{2+} binding site, in agreement with results obtained with a Ca^{2+} selective electrode [17], while the steep drop in the relaxation rate at pH 10.5 is likely to be due to dissociation into subunits. The apparent pK value of 8.0 may reflect the titration of a basic side chain or of a carboxylate group in a cluster of negative charges. In this connection it may be recalled that Ca^{2+} has a strong preference for oxygen donors in complex formation [22].

In conclusion the cation binding sites of Panulirus hemocyanin are characterized by relatively fast chemical exchange, local mobility and binding constants of the order of 10^3 or 10^4 M^{-1} for both Ca^{2+} and Mg^{2+} and 10^2 for Na^+ . These features and the pH dependence of the binding process are consistent with the binding of cations on the surface of the molecule to carboxylate groups, some of which occur in clusters. It can be calculated that the stronger binding sites will always be saturated at the physiological concentration of divalent cations ($\sim 10 \text{ mM Ca}^{2+}$ and $\sim 50 \text{ mM Mg}^{2+}$). These sites are likely to be important for the maintenance of the quaternary structure while the weaker sites will be relevant to the regulation of the HC function.

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TABLE I. Binding parameters for HC:

Method	Sites observed	No of sites/subunit	K_{Na} (M^{-1})	K_{Ca} (M^{-1})	K_{Mg} (M^{-1})	τ_c
^{23}Na NMR	"strong sites"	1	$1 \cdot 10^2$	$3 \pm 1 \cdot 10^4$	$10 \pm 2 \cdot 10^3$	16 ± 4
^{43}Ca NMR	"weak sites"	10 ± 7	--	$1 - 10 \cdot 10^3$	$\approx K_{Ca}$	14 ± 2

FIGURE LEGENDS

Figure 1. a) The full lines represent the apparent relaxation rates, calculated from a fit of a simple exponential decay (for R_1) to relaxation parameters obtained numerically and from the half width of the actual superpositions of Lorentzian lines (for R_2). In the calculations above the effect of the finite dead time (300 μ s) is taken into account [10]. The dotted lines represents the analytical expressions for the average relaxation rates, Equation (6) and (7). All data were calculated using $\omega = 1.078 \cdot 10^8 \text{ s}^{-1}$ and $\chi = 1 \text{ MHz}$
 b) The ratio R_2/R_1 as a function of $\omega\tau_C$. The data were calculated as described above.

Figure 2. The protein concentration dependence of the apparent ^{43}Ca transverse relaxation rate for a 1.0 mM CaCl_2 solution, pH 6.9. The data were recorded at 23°C . The solid line represents the model of Equation (11) and (12) using $n=5$, $K_{\text{Ca}}^W = 4 \cdot 10^3 \text{ M}^{-1}$ and $R_2^B = 8 \cdot 10^2 \text{ s}^{-1}$. The data allows different sets of parameters (R_2^B fixed) from $K_{\text{Ca}}^W = 1 \cdot 10^3 \text{ s}^{-1}$, $n = 16$ to $K_{\text{Ca}}^W = 1 \cdot 10^4$, $n = 4$.

Figure 3. The apparent ^{43}Ca transverse relaxation rate for a 0.33 mM HC solution, pH 7.2 and at 23°C , as a function of the total concentration of added CaCl_2 . The model of Equations (11) and (12) was fitted to the data, yielding the following parameters (solid line): $R_2^B = 9 \cdot 10^2 \text{ s}^{-1}$, $K_{\text{Ca}}^W = 1 \cdot 10^3 \text{ M}^{-1}$ and $n = 3$.

Figure 4. The temperature dependence of the apparent transverse excess ^{43}Ca relaxation rate for a 1.0 mM CaCl_2 solution containing (●) 0.33 mM HC at pH 6.6 and (■) 0.15 mM HC at pH 9.4.

Figure 5. The pH dependence of the apparent transverse excess ^{43}Ca relaxation rate for a 1.0 mM CaCl_2 solution containing 0.11 mM HC. The data were recorded at 23°C . The dotted line represents the model of two single protonation steps having the apparent pK values: 5.3 and 8.0.

Figure 6. The dependence of the apparent ^{43}Ca excess transverse relaxation rate for a 0.8 mM CaCl_2 solution, pH 6.7, containing 0.43 mM HC, on the total concentration of added MgCl_2 . The solid line represents a model with Ca^{2+} and Mg^{2+} competing for the same sites with approximately the same affinities: $K_{\text{Ca}}^W = 1 \cdot 10^3 \text{ M}^{-1}$ and $K_{\text{Mg}}^W = 8 \cdot 10^2 \text{ M}^{-1}$.

Figure 7a. The excess transverse relaxation rate of the fast decaying component of the ^{23}Na NMR spectrum for a 12.0 mM NaCl solution, pH 6.7, containing 0.14 mM HC as a function of added Ca^{2+} (23°C). The solid line represents the model: $R_{2,1}^{\text{EX}} = p_B R_{2,1}^{\text{B}} + C$. (The value of p_B was numerically computed using the model of Equation (9) and (10) assuming $K_{\text{Na}}^S = 1 \cdot 10^2 \text{ M}^{-1}$ [7]) where $n = 0.7$, $K_{\text{Ca}}^S = 3 \cdot 10^4 \text{ M}^{-1}$ and $R_{2,1}^{\text{B}} = 10^5 \text{ s}^{-1}$ ($C = 40 \text{ s}^{-1}$ and is experimentally obtained as an asymptote at high Ca^{2+} concentration).

Figure 7b. The excess transverse relaxation rate $R_{2,1}^{\text{EX}}$ of the ^{23}Na NMR spectrum of a 12.0 mM NaCl solution, pH 6.7, containing 0.14 mM HC. The data were measured at 23°C . The model presented in the caption to Figure 2 was fitted to the relaxation data assuming $K_{\text{Na}}^S = 1 \cdot 10^2 \text{ M}^{-1}$ and $n = 0.7$. This gave in $K_{\text{Mg}}^S = 1 \cdot 10^4 \text{ M}^{-1}$ and $R_{2,1}^{\text{B}} = 10^5 \text{ s}^{-1}$ ($C = 40 \text{ s}^{-1}$). The agreement between the fitted model and the experimental data is shown as the solid line.

Figure 1.

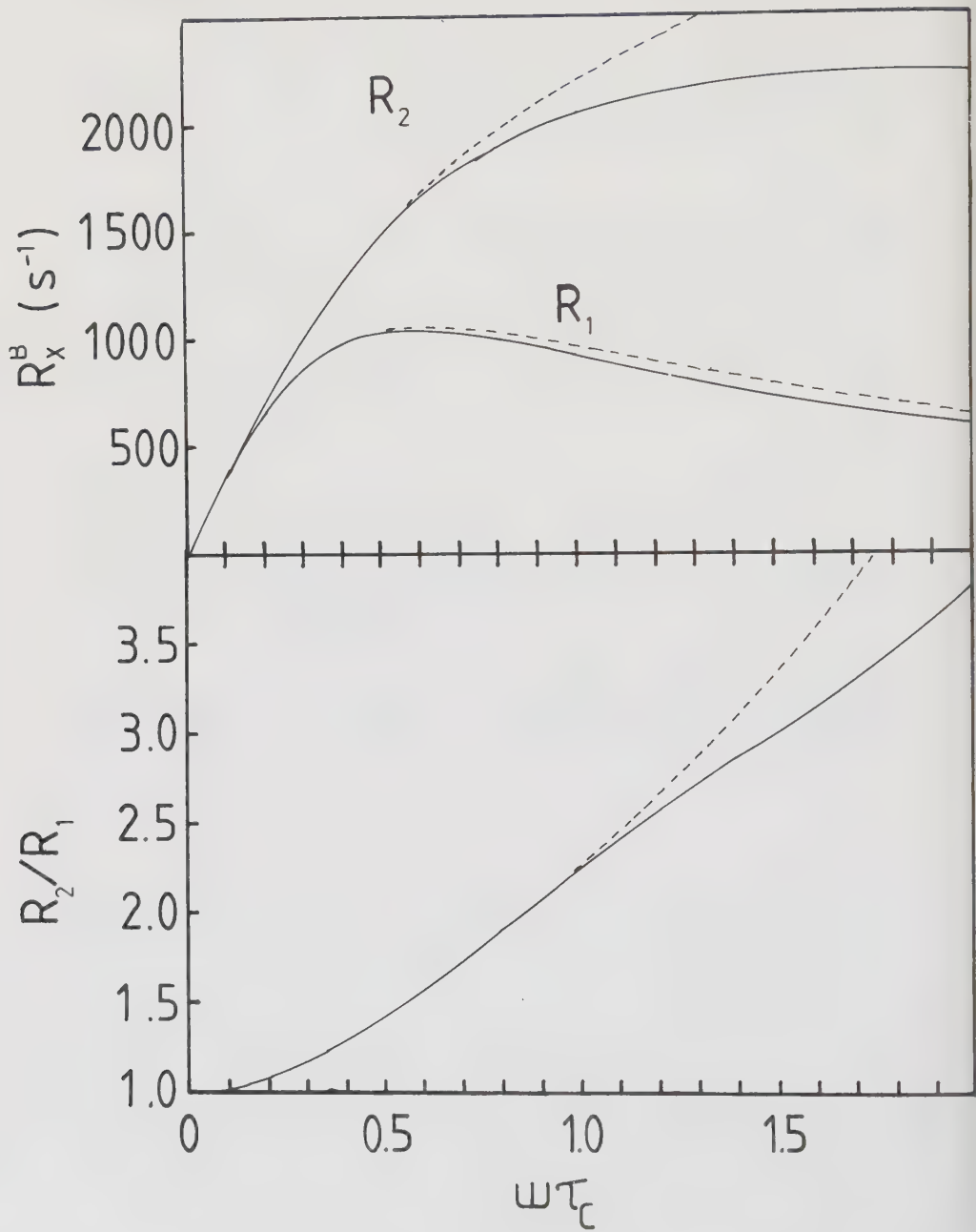


Figure 2

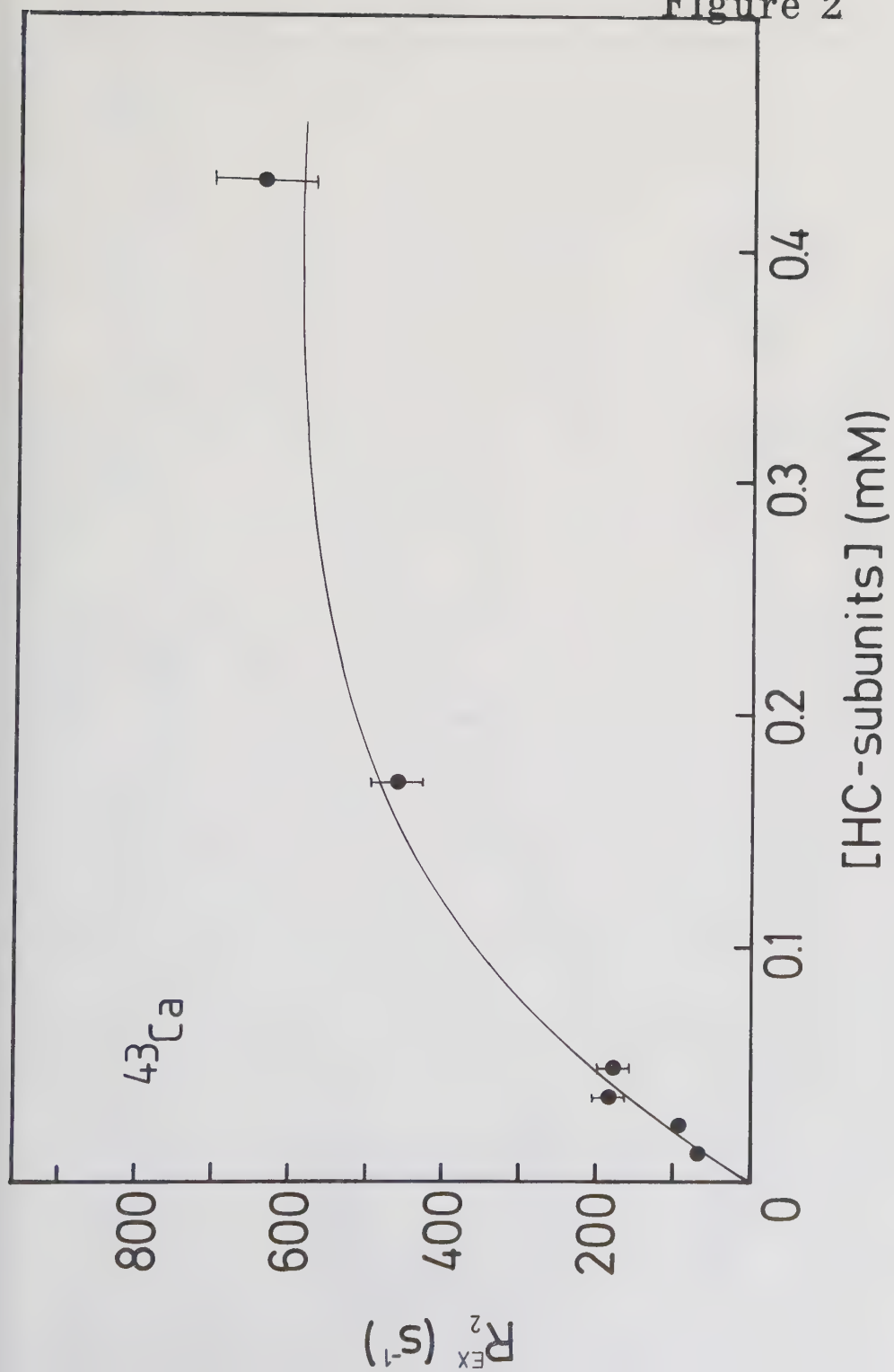


Figure 3.

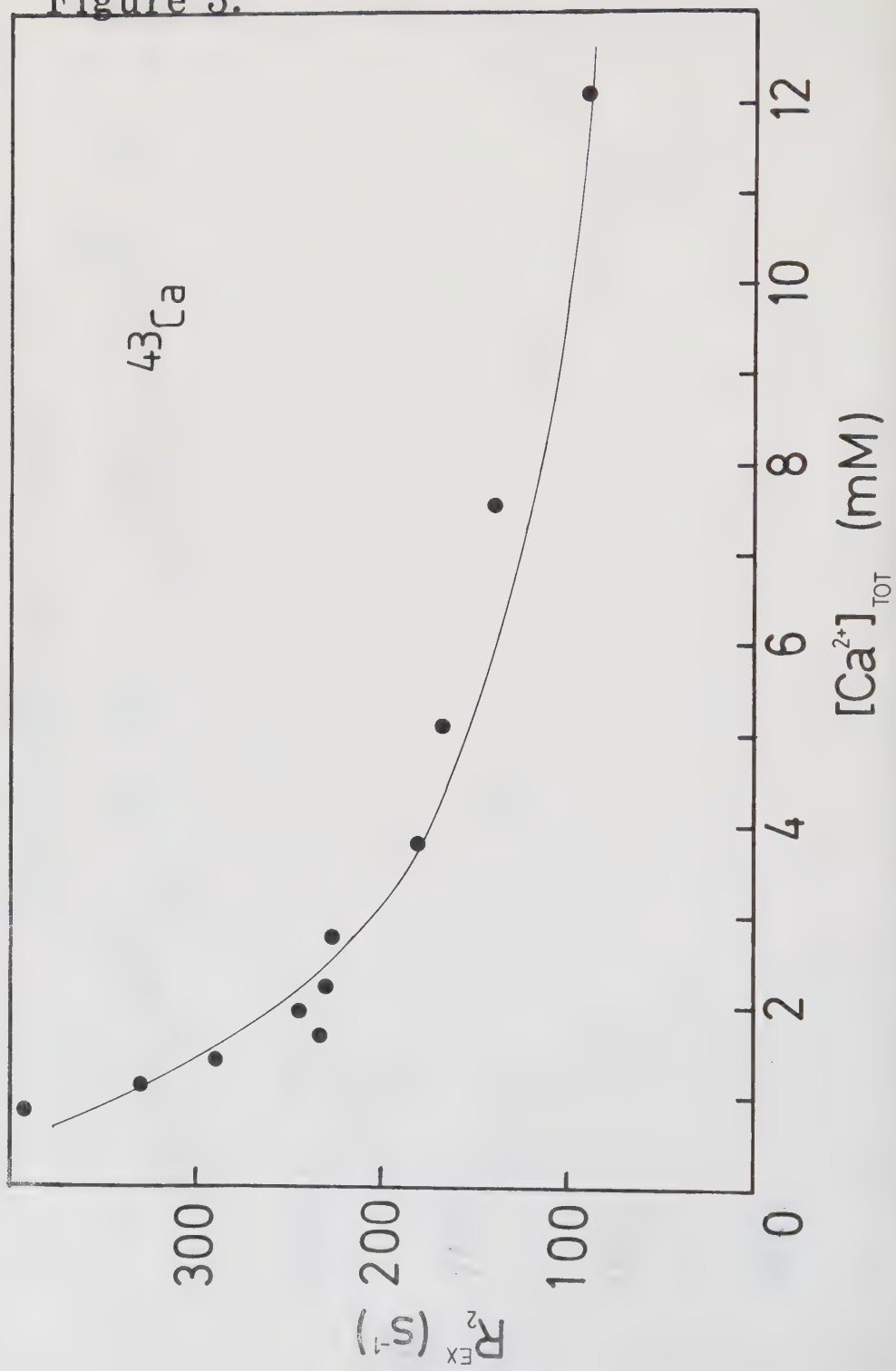


Figure 4.

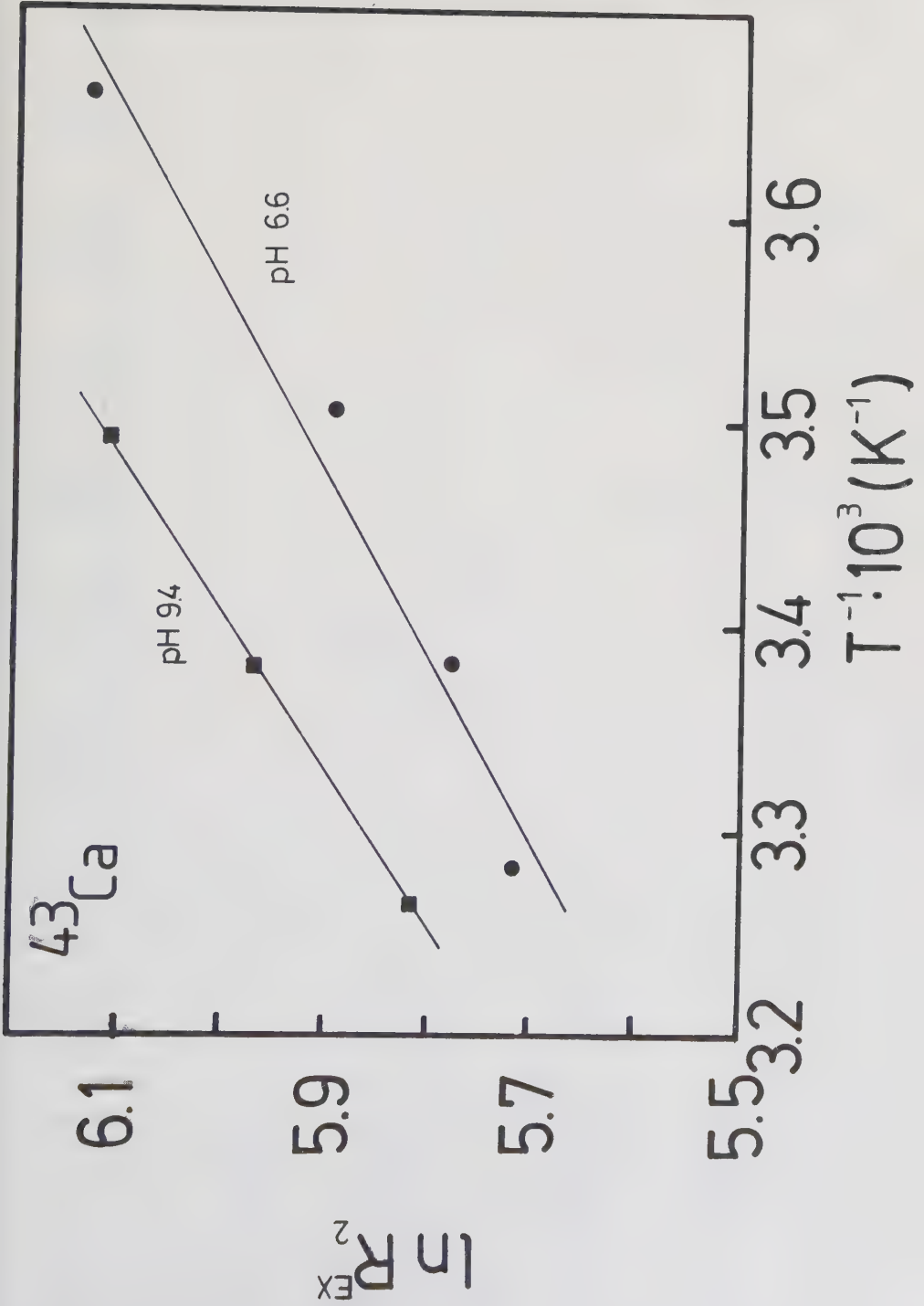


Figure 5.

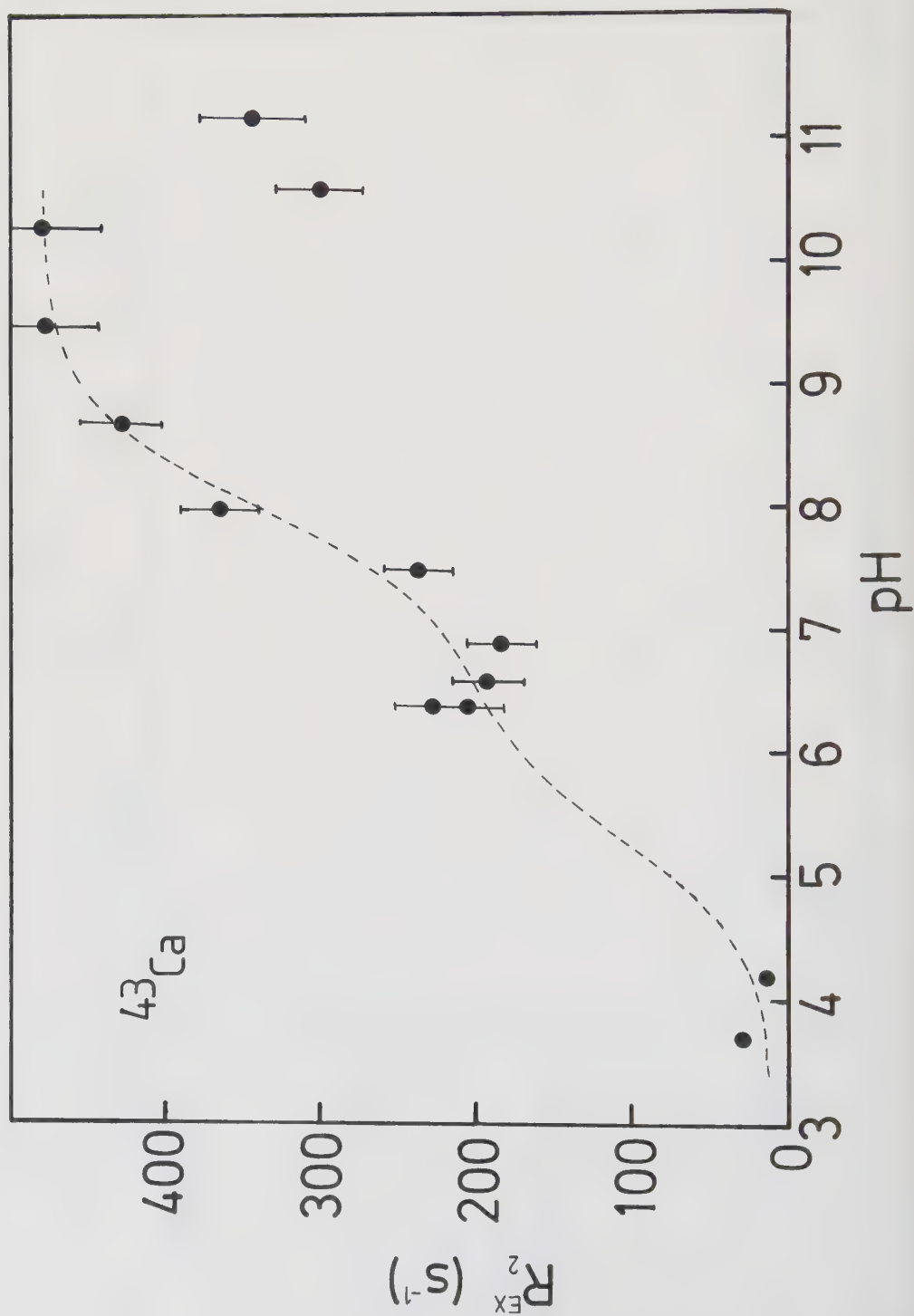


Figure 6.

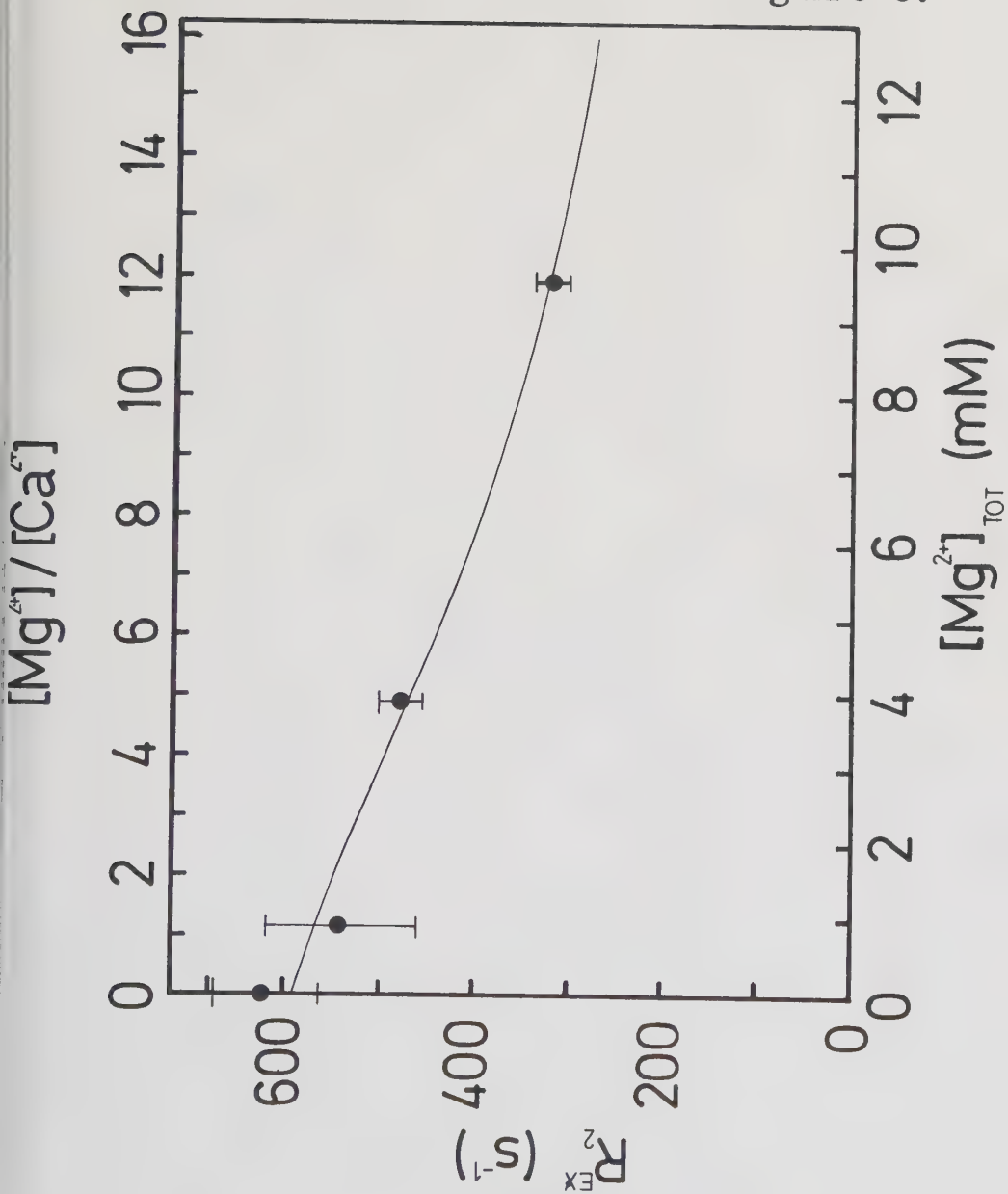


Figure 7.

